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(57) Abstract

Malto-oligosaccharide (MOS) content of cells (particularly plants cells) is modulated, resulting in alterated α-glucan (e.g. starch) production by the cells. Granule Bound Starch Synthase I (GBSSI) activity in production of unbranched α-glucan, such as amylose, and branched α-glucan, such as amylopectin, is modified by modulating MOS content.

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MODIFIED PLANTS AND PLANT PRODUCTS

The present invention relates to plant modification, particularly modification via the manipulation of activity of a plant enzyme "GBSSI" an α-glucan synthase to alter the nature of starch an α-glucan produced by the plant. GBSSI activity is modulated by controlling malto-oligosaccharide content, found to be required for amylose (an unbranched α-1,4 glucan with few or no α-1,6 linkages) synthesis by GBSSI. When the availability of malto-oligosaccharides to GBSSI is reduced the enzyme extends amylopectin (a branched α-1,4, α-1,6 glucan) instead of amylose.

Starch granules from storage organs such as cereal endosperms, potato tubers and pea embryos contain two or more distinct isoforms of starch synthase. All have a highly-conserved, exclusively granule-bound isoform of about 60 kDa - usually the major granule-bound protein - which will be referred to as granule-bound starch synthase I (GBSSI), and one or more other isoforms. Analysis of mutants lacking GBSSI has shown that this isoform is specifically required for the synthesis of amylose, the relatively unbranched glucan polymer which comprises approximately 30% by weight of the starches of wild-type storage organs (Smith and Martin 1993). Thus, it is accepted that GBSSI (also known as "the waxy protein") is responsible for synthesis of the amylose

component of starch (evidence reviewed in Smith and Martin 1993, Smith et al. 1995, Martin and Smith 1995).

The function of the GBSSs other than those of the GBSSI class has not been established. It is clear from studies of starches of mutants that lack GBSSI that these other isoforms cannot synthesise amylose to any significant extent (eg Denyer et al. 1995a, Hylton et al. 1995). However, it is not known whether these isoforms contribute to the synthesis of amylopectin within the granule. Some of the GBSSs other than GBSSI, such as the 77-kDa GBSS of developing pea embryos (referred to as GBSSII), are found in the soluble fraction of the amyloplast as well as being associated with starch granules (Denyer et al. 1993). Such isoforms are very likely to be involved in amylopectin synthesis when soluble, and may become trapped within the matrix of the granule as it grows. Other GBSSs appear to be predominantly if not entirely associated with starch granules, such as the 100-105-kDa GBSS of developing wheat endosperm (Denyer et al. 1995b). Thus, at present there is no evidence to suggest that the other GBSSs do participate in the synthesis of amylose (Hylton et al. 1995, Denyer et al. 1995a). Amylose synthesis may be an exclusive property of the waxy protein.

In spite of this widely accepted hypothesis, no-one has demonstrated amylose synthesis by an isolated

starch granule from a higher plant. It has been established that glucose from ADPglucose (ADPG) is readily incorporated into the starch of storage organs of crop plants when ADPG is supplied to isolated starch granules (eg. Frydman and Cardini 1967, Macdonald and Preiss 1983, Smith 1990, Hylton et al. 1995, Denyer et al. 1995b). However, this incorporation is into a amylopectin and not an amylose (Baba et al. 1987).

The present invention is based on the surprising discovery that glucan of low molecular weight (malto-oligosaccharides: referred to as MOS) present *in vivo* but apparently not in isolated starch granules is required for amylose synthesis by GBSSI. Without malto-oligosaccharide GBSSI extends amylopectin, not amylose.

Evidence for this is described in detail herein, but to summarise:

1. Isolated starch granules from developing embryo of pea and developing potato tuber incorporate glucose from ADPG mainly into branched α-glucan chains. Very little glucose is incorporated into amylose.

2. Both the GBSSI and other GBSS isoforms contribute to this synthesis of amylopectin. Starch granules isolated from the developing embryos of mutant peas which completely lack GBSSI protein and amylose, but retain a second GBSS isoform, (*lam*

mutant: Denyer et al 1995a) incorporate glucose from ADPG into amylopectin, but at a much lower rate on a starch basis than starch from wild-type peas. This indicates that a considerable proportion of the amylopectin synthesis by isolated starch granules from wild-type pea embryos is via GBSSI.

3. Addition of MOS to isolated starch granules of wild-type pea embryos and potato tubers causes an increase in the amount and a substantial shift in the pattern of incorporation of glucose from ADPG. Incorporation into amylopectin is reduced, and incorporation into lower molecular weight material of the same mobility as amylose on gel-permeation columns is substantially increased. MOS ranging from maltose (G2) to malto-heptaose (G7) have been shown to cause this shift. Glucose does not have this effect.

4. The synthesis of amylose from ADPG in the presence of MOS is exclusively a function of GBSSI. MOS had no effect on the incorporation of glucose from ADPG into isolated starch granules of the lam mutant that lacks GBSSI: incorporation was into amylopectin whether MOS were present or not.

5. Extracts of the storage organs of higher plants contain compounds that mimic the effect of MOS on the incorporation of glucose from ADPG into isolated starch granules. When ADPG is supplied to a homogenate of pea or potatoes, glucose is incorporated into both amylopectin and amylose within the granules

in the homogenate. Rapid washing of granules from the homogenate to remove soluble components of the homogenate lowers the total rate of incorporation of glucose from ADPG, and results in loss of incorporation into amylose. Addition of the heat-stable soluble fraction of the homogenate to isolated granules promotes incorporation of glucose from ADPG and causes a shift from amylopectin towards amylose synthesis, in the same manner as the addition of MOS.

6. The compounds in plant extracts that mimic the effects of MOS are themselves MOS. Incubation of the heat-stable soluble fraction of homogenates of potato tuber with α-glucosidase - an enzyme that will specifically convert MOS to glucose - destroys the ability of this fraction to stimulate incorporation of glucose from ADPG into isolated starch granules, and to redirect synthesis from amylose to amylopectin.

The results indicate that the presence of MOS in the amyloplast *in vivo* is a prerequisite for the synthesis of amylose via GBSSI and that GBSSI activity is redirected from amylose to amylopectin in the absence of MOS. Accordingly, the present inventors have realised that manipulation of MOS levels *in planta* provides a means for manipulation of starch produced by the plant, in particular the nature of the amylopectin component of the starch.

The implications and applications of this work

are many-fold. Manipulation of the level of MOS in amyloplasts of the storage organ of a plant will affect amylose synthesis by GBSSI and action of GBSSI on chains of amylopectin. Accordingly, the properties of the starch produced by the plant will be modified. There is considerable interest in modifying starch and creating and investigating novel properties. Starch is of major interest in many industries, including the food, paper, oil, glue, tobacco and textile industries. For instance, starch, modified or otherwise, is used in beer, animal feeds, baking products such as biscuits, doughnuts, pies, crisps, in canned foods such as soups, fruits and vegetables, in cereals, in condiments such as ketchup, in fats and oils such as margarine, in dried foods, syrups and sweeteners, in a whole host of other foods, in building materials such as cardboard, ceramics, fiberboard and fiberglass, paints, in numerous chemical products and processes, in crayons and chalk, cord, string, in metallurgy, explosives, rubber, textiles, dyes and in tobacco. Potato starch, for example, is used for sizing and surface coating in the paper industry, in sizing of cotton, worsted and spun rayon wraps, and in finishing sewing thread and cloth in the textile industry.

"Waxy corn", in which the starch is composed entirely of amylopectin (a branched α-glucan) as compared with 72 percent amylopectin and 28 percent

amylose (an unbranched α-glucan) in common corn starch. Several alleles have been observed at the waxy locus. See, for example, "CORN - Improvement, Seed Production and Uses", Jugenheimer (1976), John Wiley & Sons, USA, for a discussion.

A group including Visser at the Agricultural University and CPRO Institute in Wageningen, The Netherlands, have used anti-sense technology targeting waxy protein expression to produce a waxy starch potato. This work was commissioned by the paper industry, providing further illustration of the interest in starch modification and its importance.

According to a first aspect of the present invention there is provided a method comprising modulating the amount of malto-oligosaccharide (MOS) present in a plant cell. Specifically amyloplasts of a storage organ of a plant are of interest. Altering the level of malto-oligosaccharide in the cell affects GBSSI production of amylose and amylopectin thereby influencing the nature of the starch produced.

In one embodiment of the present invention, malto-oligosaccharide in the cell is depleted. Any mechanism that reduces or eliminates MOS from amyloplasts of a developing storage organ prevents or reduces amylose synthesis by GBSSI. GBSSI action upon the chains of amylopectin in the granule is concomitantly increased. This results in a starch which lacks or has reduced levels of amylose, and has

an amylopectin structure that is different from that of both wild-type starches and the starch of mutant and transgenic plants which lack or have very low levels of GBSSI (for example the *waxy* mutants of maize, barley, sorghum rice and *Amaranthus*, the *amf* mutant of potato, the *lam* mutant of pea, and transgenic potatoes expressing an antisense construct containing any GBSSI sequence: specific literature references in Smith and Martin 1993) and may be different from that of wild-type starch. The amylopectin of plants which lack or have very low levels of MOS should have longer branches than amylopectin from wild-type plants or plants with altered levels of GBSSI of the same species, and may have longer branches than the amylopectin from wild-type plants of the same species.

Thus an aspect of the present invention provides a method of modifying amylopectin produced in a plant cell (or a method of producing modified amylopectin in a plant cell), the method comprising modulating the amount of malto-oligosaccharide (MOS) in the cell. Altering the MOS level within the cell affects the action of GBSSI, and thus alters the amylose and amylopectin contents of the cell. In preferred embodiments, MOS is depleted (wholly (which includes substantially wholly) or partially) within the cell, leading to modified amylopectin production. The use of a substance which reduces the level of malto-

oligosaccharide in a plant cell in modifying starch produced in the cell (especially the amylopectin component of the starch) is provided as a further aspect of the present invention.

The level of MOS in a plant cell may be assessed quantitatively by the following method. First, the metabolism of the cell is stopped by extremely rapidly. Typically, the tissue is clamped in tongs cooled to the temperature of liquid nitrogen, then allowed to thaw in 1 M perchloric acid. A soluble extract of the tissue at neutral pH suitable for enzymic assay of metabolites is then prepared. In the example above, the perchloric acid extract of the tissue is brought to neutrality with a suitable alkaline solution such as 5 M potassium carbonate, and the resulting insoluble potassium perchlorate is removed by centrifugation. The extract is incubated with enzymes and other factors required to remove sucrose and glucose. This may be achieved, for example, by the addition of invertase, glucose 6-phosphate dehydrogenase, adenosine triphosphate, nicotinamide adenine dinucleotide, and magnesium chloride at appropriate concentrations as described by Lowry and Passonneau (1971). The extract is then incubated with an excess of α-glucosidase and α-amyloglucosidase to hydrolyse MOS to glucose, and the glucose is assayed for example as described by Lowry and Passonneau (1971). The amount and nature of the

MOS in a tissue may be investigated by several methods utilising a fraction containing soluble neutral compounds obtained from an extact (prepared as described above) by ion-exchange chromatography. These methods include thin-layer chromatography, paper chromatography, and high-performance anion-exchange chromatography with pulsed amperometric detection.

Partial or total elimination of MOS in an amyloplast may be achieved using an enzyme that uses MOS as a substrate, converting it into something else. For example, suitable enzymes convert MOS to glucose. One way of providing such an enzyme, or other substance that reduces MOS availability to GBSSI, to a plant cell is by expression within the cell of an encoding nucleic acid sequence. A preferred enzyme for this purpose is an α-glucosidase (EC 3.2.1.20), which may have a strong maltase activity (ie a low Km for maltose) and possibly little or no isomaltase activity (ie little or no action on α-1,6 linkages) or activity with starch granules. Obviously, it is preferred that the enzyme has a pH optimum within the range of pH likely to occur within the cytoplasm of the cell (typically 6.5-7.8).

Enzymes with such activity may be identified by their ability to hydrolyse the specific substrate 4-nitropenyl glucoside, as described by Needleman et al. (1978). Incubation of the enzyme at an appropriate pH and with a suitable concentration of the substrate

results in the appearance of a yellow colour as hydrolysis proceeds.

The Km for maltose or any other MOS may be defined by the incubation of the enzyme at a suitable pH with a range of concentrations of maltose or other MOS for a defined period over which the reaction has been established to proceed linearly. Measurement of the amount of glucose produced over the defined period by any suitably specific and sensitive means (for example by enzymic assay as described by Lowry and Passonneau, 1971) then provides a quantitative estimate of enzyme activity from which the affinity of the enzyme for its substrate may be calculated by any of the methods described for this purpose by Cornish-Bowden (1995).

The pH optimum of the enzyme may be discovered by incubation of the enzyme with a suitable substrate at a range of different pH (typically a range from 5.5 to 8.5) followed by measurement of the amount of substrate hydrolysed at each pH. The action of the enzyme upon starch may be examined by the measurement of the production of reducing sugars upon incubation of the enzyme with starch granules, or with solubilised polymers derived therefrom. A suitable method for the measurement of reducing sugars is described by Bernfeld (1951).

All of the above methods may be applied to purified or partially purified enzymes obtained from a

commercial source or by purification or partial purification from any appropriate organism. Methods of purification are exemplified by that described for yeast by Needleman et al. (1978). The techniques described above may also be used to study enzymes in cell extracts prepared from any suitable organism. However, any person skilled in the art will appreciate that cell extracts may contain several activities capable of the hydrolysis of MOS and/or starch, and definition of the properties of any one of these activities may not be possible without partial purification.

The maltase of the bacterium *Bacillus amylolyticus* and the maltase encoded at the *MAL6* locus of the yeast *Saccharomyces carlsbergensis* are suitable (Kelly and Fogarty 1983): the gene for the *Saccharomyces* maltase has been cloned (Hong and Marmur 1986).

As necessary, any person skilled in the art may clone the gene for any desired maltase from a bacterium by, for example, an obvious modification of the *E. coli* transformation method used to clone an oligo-1,6-glucosidase from *Bacillus cereus* (Watanabe et al. 1990) or by complementation of a *Saccharomyces* strain carrying mutations in structural genes for maltase and thus unable to utilise maltose as a carbon source (Federoff et al. 1982). The efficacy of α-glucosidase in destroying plant MOS that promote

amylose synthesis, thus allowing amylopectin synthesis rather than amylose synthesis by GBSSI, is demonstrated by experiments described in detail herein.

Genes may be cloned using information from sequences available in databases known to those skilled in the art. A method of obtaining nucleic acid may comprise hybridisation of an oligonucleotide or a nucleic acid molecule comprising such an oligonucleotide to target/candidate nucleic acid. Target or candidate nucleic acid may, for example, comprise a genomic or cDNA library obtainable from an organism known to contain or suspected of containing such nucleic acid. Successful hybridisation may be identified and target/candidate nucleic acid isolated for further investigation and/or use.

Hybridisation may involve probing nucleic acid and identifying positive hybridisation under suitably stringent conditions (in accordance with known techniques) and/or use of oligonucleotides as primers in a method of nucleic acid amplification, such as PCR. For probing, preferred conditions are those which are stringent enough for there to be a simple pattern with a small number of hybridisations identified as positive which can be investigated further. It is well known in the art to increase stringency of hybridisation gradually until only a few positive clones remain.

As an alternative to probing, though still employing nucleic acid hybridisation, oligonucleotides designed to amplify DNA sequences may be used in PCR reactions or other methods involving amplification of nucleic acid, using routine procedures. See for instance "PCR protocols; A Guide to Methods and Applications", Eds. Innis *et al*, 1990, Academic Press, New York.

On the basis of amino acid sequence information oligonucleotide probes or primers may be designed, taking into account the degeneracy of the genetic code, and, where appropriate, codon usage of the organism from the candidate nucleic acid is derived.

An oligonucleotide for use in nucleic acid amplification or probing may have about 10 or fewer codons (e.g. 6, 7 or 8), i.e. is about 30 or fewer nucleotides in length (e.g. 18, 21 or 24).

Assessment of whether or not such a PCR product corresponds to the gene of interest may be conducted in various ways. A PCR band from such a reaction might contain a complex mix of products. Individual products may be cloned and each one individually screened. It may be analysed by transformation to assess function on introduction into a plant of interest.

Nucleotide sequences employed in the present invention may encode a wild-type sequence (e.g. gene) selected from those available, or a mutant,

derivative, variant or allele, by way of insertion, addition, deletion or substitution of one or more nucleotides, of such a sequence. An alteration to or difference in a nucleotide sequence may or may not be reflected in a change in encoded amino acid sequence, depending on the degeneracy of the genetic code. In any case, mutants, derivatives and alleles useful in the present invention are those which retain a functional characteristic of the polypeptide encoded by the wild-type gene, i.e. maltase activity as discussed. Of course, changes to the nucleic acid which make no difference to the encoded amino acid sequence. are included.

The nucleic acid may be in the form of a recombinant vector, for example a plasmid or agrobacterium binary vector (Van den Elzen et al., 1985). The nucleic acid may be under the control of an appropriate promoter and regulatory elements for expression in a plant cell. In the case of genomic DNA, this may contain its own promoter and regulatory elements and in the case of cDNA this may be under the control of an appropriate promoter and regulatory elements for expression in the host cell.

Those skilled in the art are well able to construct vectors and design protocols for recombinant gene expression. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator

fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual:* 2nd edition, Sambrook *et al,* 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Short Protocols in Molecular Biology,* Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. The disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference. Specific procedures and vectors previously used with wide success upon plants are described by Beran (1984) and Guerineau and Mullineaux (1993).

Selectable genetic markers may be used consisting of chimaeric genes that confer selectable phenotypes such as resistance to antibiotics such as kanamycin, hygromycin, phosphinotricin, chlorsulfuron, methotrexate, gentamycin, spectinomycin, imidazolinones and glyphosate (Herrera-Estrella *et al,* 1983; van den Elzen *et al,* 1985).

Introduction or elevation of activity of α-glucosidase into amyloplasts of the developing storage organs of crop plants may be achieved using any appropriate method of plant transformation to generate

plant cells comprising nucleic acid with a sequence encoding the enzyme. Plants may be regenerated from transformed plant cells and tissue.

For example, potato may be transformed by incubation of tuber slices with Agrobacterium cells carrying the gene of interest and a gene encoding resistance to kanamycin in a suitable construct (Spychalla and Bevan, 1993). After co-cultivation for two days on tobacco feeder layers, discs are transferred onto Murashige and Skoog (MS) medium containing cefotaxime to select against Agrobacterium, then transferred to agar plates containing MS medium, cefotaxime, and kanamycin to select for growth of transformed cells. Shoots are excised from the discs and subcultured twice on MS medium containing kanamycin before transfer to soil.

When introducing a chosen gene construct into a cell, certain considerations must be taken into account, well known to those skilled in the art. The nucleic acid to be inserted should be assembled within a construct which contains effective regulatory elements which will drive transcription. There must be available a method of transporting the construct into the cell. Once the construct is within the cell membrane, integration into the endogenous chromosomal material either will or will not occur. Finally, as far as plants are concerned the target cell type must be such that cells can be regenerated into whole

plants.

Plants transformed with the DNA segment containing the sequence may be produced by standard techniques which are already known for the genetic manipulation of plants. DNA can be transformed into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by Agrobacterium exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711 - 87215 1984), particle or microprojectile bombardment (US 5100792, EP-A-444882, EP-A-434616) microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966, Green *et al.* (1987) *Plant Tissue and Cell Culture*, Academic Press), electroporation (EP 290395, WO 8706614 Gelvin Debeyser - see attached) other forms of direct DNA uptake (DE 4005152, WO 9012096, US 4684611), liposome mediated DNA uptake (e.g. Freeman *et al. Plant Cell Physiol.* 29: 1353 (1984)), or the vortexing method (e.g. Kindle, *PNAS U.S.A.* 87: 1228 (1990d) Physical methods for the transformation of plant cells are reviewed in Oard, 1991, *Biotech. Adv.* 9: 1-11.

Agrobacterium transformation is widely used by those skilled in the art to transform dicotyledonous species. Recently, there has been substantial progress towards the routine production of stable, fertile transgenic plants in almost all economically relevant monocot plants (Toriyama, et al. (1988) *Bio/Technology* 6, 1072-1074; Zhang, *et al.* (1988)

Plant Cell Rep. 7, 379-384; Zhang, *et al.* (1988) *Theor Appl Genet* 76, 835-840; Shimamoto, *et al.* (1989) *Nature* 338, 274-276; Datta, *et al.* (1990) *Bio/Technology* 8, 736-740; Christou, *et al.* (1991) *Bio/Technology* 9, 957-962; Peng, *et al.* (1991) International Rice Research Institute, Manila, Philippines 563-574; Cao, *et al.* (1992) *Plant Cell Rep.* 11, 585-591; Li, *et al.* (1993) *Plant Cell Rep.* 12, 250-255; Rathore, *et al.* (1993) *Plant Molecular Biology* 21, 871-884; Fromm, *et al.* (1990) *Bio/Technology* 8, 833-839; Gordon-Kamm, *et al.* (1990) *Plant Cell* 2, 603-618; D'Halluin, *et al.* (1992) *Plant Cell* 4, 1495-1505; Walters, *et al.* (1992) *Plant Molecular Biology* 18, 189-200; Koziel, *et al.* (1993) *Biotechnology* 11, 194-200; Vasil, I. K. (1994) *Plant Molecular Biology* 25, 925-937; Weeks, *et al.* (1993) *Plant Physiology* 102, 1077-1084; Somers, *et al.* (1992) *Bio/Technology* 10, 1589-1594; WO92/14828). In particular, *Agrobacterium* mediated transformation is now emerging also as an highly efficient alternative transformation method in monocots (Hiei *et al.* (1994) *The Plant Journal* 6, 271-282).

The generation of fertile transgenic plants has been achieved in the cereals rice, maize, wheat, oat, and barley (reviewed in Shimamoto, K. (1994) *Current Opinion in Biotechnology* 5, 158-162.; Vasil, *et al.* (1992) *Bio/Technology* 10, 667-674; Vain et al., 1995, *Biotechnology Advances* 13 (4): 653-671; Vasil, 1996,

Nature Biotechnology 14 page 702).

Microprojectile bombardment, electroporation and direct DNA uptake are preferred where Agrobacterium is inefficient or ineffective. Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, eg bombardment with Agrobacterium coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with Agrobacterium (EP-A-486233).

Following transformation, a plant may be regenerated, e.g. from single cells, callus tissue or leaf discs, as is standard in the art. Almost any plant can be entirely regenerated from cells, tissues and organs of the plant. Available techniques are reviewd in Vasil et al., *Cell Culture and Somatic Cel Genetics of Plants, Vol I, II and III, Laboratory Procedures and Their Applications*, Academic Press, 1984, and Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, 1989.

The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant cells is not

essential to or a limitation of the invention, nor is the choice of technique for plant regeneration.

A suitable method for transforming potatoes such that the product of the introduced gene may be targeted to amyloplasts is exemplified by the work of Shewmaker et al. (1994). A plasmid pCGN1132S contains a 35S promoter, a transit peptide and 16 amino acids of the mature protein of the pea ribulose bisphosphate carboxylase small subunit gene. The gene encoding the enzyme of interest is cloned into the plasmid as an in-frame fusion with the 16 amino acids of the small subunit. The fusion gene is excised and cloned into the patatin promoter cassette pCGN2162. A DNA segment containing the 700-base pair patatin promoter, the transit peptide, the 16 amino acids of the small subunit, the gene of interest and a 300-base pair nos 3' region is excised and cloned into the binary vector pCGN1557. This plasmid is then transferred to *Agrobacterium tumefaciens* strain LBA4404 and used to transform potatoes. The efficacy of this method for achieving targeting of proteins to amyloplasts is illustrated by immunoblots which show correct processing of the expressed protein in potato tubers. The ability of the method to bring about alterations of starch structure in potatoes is illustrated by its use to introduce a gene encoding a bacterial glycogen synthase (Shewmaker et al., 1994). The amylopectin of transformed plants had fewer longer branches and more

shorter branches than control plants.

A second method which may be used to target an α-glucosidase to the amyloplasts of the storage organs of crop plants is exemplified by the work of Klösgen and Weil (1991). In a procedure similar to that described above, these workers fused DNA encoding the transit peptide and 34 amino acids of the mature waxy protein of maize to the gene encoding the ß-glucuronidase of *E. coli*. Introduction of the construct into potato plants via an *Agrobacterium* based method led to expression of the fusion protein in chloroplasts and amyloplasts of the transgenic plants.

Levels of MOS in a cells may be reduced via a reduction within that cell in the activity of one or more of the endogenous enzymes capable of generating MOS. Such enzymes, and methods for their identification and the cloning of genes that encode them, are discussed elsewhere herein. Reductions in activity may be achieved by the introduction into the cell of a gene - or part thereof - in the antisense orientation, such that an antisense RNA is produced within the cell. The gene or part thereof will encode the endogenous enzyme or another enzyme sufficiently similar in sequence to the gene encoding the endogenous enzyme. The introduction of the gene may be achieved by any suitable transformation method, as discussed elsewhere herein. Development and use of

constructs containing genes in the antisense orientation to bring about a reduction in an enzyme activity is exemplified by the work of Edwards et al. (1995)and Marshall et al. (1996), who employed this technology to bring about large reductions in specific forms of starch synthase within the potato plant.

A further aspect of the invention provides a starch-producing cell of a plant, the level of malto-oligosaccharide in the cell being reduced compared with the level in an equivalent wild-type cell and the starch having an increased or modified amylopectin component compared with the starch produced by the equivalent wild-type cell. Preferably, the level of malto-oligosaccharide is reduced by virtue of expression within the cell of a polypeptide with maltase activity (as disclosed) from heterologous encoding nucleic acid therefor.

The term "heterologous" indicates that the gene/sequence of nucleotides in question has been introduced into cells of the plant or an ancestor thereof, using genetic engineering, i.e. by human intervention. The gene may be on an extra-genomic vector or incorporated, preferably stably, into the genome.

The cell may comprise the nucleic acid encoding the enzyme by virtue of introduction into the cell or an ancestor thereof of the nucleic acid, e.g. by

transformation, using any suitable technique available to those skilled in the art. Furthermore, plants which comprise such cells, and seed therefor, may be produced by crossing suitable parents to create a hybrid whose genome contains the required nucleic acid, in accordance with any available plant breeding technique.

Also according to the invention there is provided a starch-producing plant cell as disclosed having incorporated into its genome a sequence of nucleotides encoding a polypeptide with maltase activity under operative control of a regulatory sequence for expression of the encoded polypeptide. Expression of the polypeptide results in depletion of malto-oligosaccharides in the cell, which in turn results in action of GBSSI on amylopectin as opposed to amylose to produce modified amylopectin. A further aspect of the present invention provides a method of making such a plant cell involving introduction of a vector comprising the sequence of nucleotides into a plant cell. Such introduction may be followed by recombination between the vector and the plant cell genome to introduce the sequence of nucleotides into the genome. The polypeptide encoded by the introduced nucleic acid may then be expressed in the cell and descendants thereof, including cells in plants regenerated from transformed material. A gene stably incorporated into the genome of a plant is passed from

generation to generation to descendants of the plant, so such decendants should show the desired phenotype.

The present invention also provides a plant comprising a plant cell as disclosed. Such a plant produces modified amylopectin and starch as disclosed. A plant according to the present invention may be one which does not breed true in one or more properties. Plant varieties may be excluded, particularly registrable plant varieties according to Plant Breeders' Rights. It is noted that a plant need not be considered a "plant variety" simply because it contains stably within its genome a transgene, introduced into a cell of the plant or an ancestor thereof.

In addition to a plant, the present invention provides any clone of such a plant, seed, selfed or hybrid progeny and descendants, and any part of any of these, such as cuttings, seed. The invention provides any plant propagule, that is any part which may be used in reproduction or propagation, sexual or asexual, including cuttings, seed and so on.

The present invention is particularly beneficial for use in plants such as crop plants, including cereals and pulses, maize, wheat, potatoes, tapioca, rice, sorgum, millet, cassava, barley, pea and other root, tuber or seed crops, in principle any plant which is a source of starch.

The amylopectin and/or starch may be extracted

from plants according to the invention following its production in cells of the plant. Starch and/or amylopectin may be obtained using any available tecnique. For instance, potato starch is easily separated from tubers and in early nineteenth-century households was commonly prepared for domestic use. On an industrial scale, starch may be obtained from potatoes using, for example, a method along the following lines: The potatoes are disintegrated in a stream of water in a rapidly rotating vertical hammer-mill enclosed by a cylindrical perforated screen. Through this the pulp is forced and passes into a series of centrifugal rotating sieves. The starch suspension which has passed through the sieves proceeds, with the addition of wash water, through a number of centrifugal separators to remove soluble impurities, then through a vacuum filter, a preliminary conveyor-drier with a countercurrent of air at about 145 °C, followed by a series of blowers with cyclone separators, also at 145 °C, and a final cool-air blower with cyclone.

A further aspect of the present invention thus provides starch or amylopectin obtained or obtainable from a plant or plant cell according to the invention as disclosed.

The starch or amylopectin removed from a plant may be purified and may be formulated into a composition. A composition containing such starch or

amylopectin may include one or more, i.e. at least one, additional component. Such compositions include food formulations, industrial formulations etc. Uses of starches and compositions containing starch have been discussed above.

"Malto-oligosaccharide" includes individually or collectively any α-1,4 or α-1,4,α1,6 glucan that is soluble within the cell rather than forming part of a polymer within the starch granule, including each of the mono- and oligomers investigated experimentally herein, such as maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and/or maltoheptaose.

Reference to malto-oligosaccharide "content" is to composition (quality) and/or level (quantity).

It should be noted that terms such as "modulating" and "altering" are used in relation to control being exercised over something as a result of human intervention. With respect to the level of MOS in a cell, the intervention may result in an increase or decrease in the level of MOS as compared with the level in the absence of the intervention, i.e. in comparison with equivalent wild-type cells, e.g. of plants of the same species. (Cells which are wild-type in respect of MOS levels may of course not be wild-type in every respect.)

Instead of modulating the level of MOS in a cell by way of reduction or depletion, the level may be increased in order to direct action of GBSSI from

amylopectin to amylose.

Any mechanism that increases the concentration of MOS in the amyloplast of developing storage organs may bring about an increased synthesis of amylose by increasing the availability of primer for GBSSI activity or the affinity of GBSSI for amylose relative to amylopectin within the starch granule. This results in an increased ratio of amylose to amylopectin in the starch, and may also affect the size and/or structure of amylose molecules. An increased concentration of MOS may be brought about by the introduction into the amyloplast of any enzyme capable of the generation of MOS.

These glucans can be generated *in vivo* by several enzymes known to be present in developing starch-storing organs, namely ß-amylase, α-amylase, disproportionating enzyme, debranching enzyme and starch phosphorylase. Glucans can also be generated by other enzymes, including debranching enzymes, starch phosphorylases and amylosucrases of bacteria. The first four of these enzymes can generate glucans by cleavage or rearrangement of nascent glucan chains synthesised by starch synthase and starch-branching enzyme. Starch phosphorylase can generate glucans from glucose 1-phosphate (Steup 1988, 1990; Takaha et al. 1993).

Typically preferred enzymes for use in this aspect of the invention have low or negligible

activity against intact starch granules, and may be a starch phosphorylase or a ß-amylase. Obviously, a pH optimum within the range of pH likely to occur within the all cytoplasm (typically 6.5-7.8) is preferred. Methods for identification and characterisation of activities of these enzymes are extensively discussed by Steup (1990). Genes for plastidial isoforms of phosphorylase, α-amylases, β-amylases, disproportionating enzymes and debranching enzymes have been cloned (Nakano et al. 1989, Lin et al. 1991, Sonnewald et al. 1995, Yoshida et al. 1992, Siggens 1987, Monroe et al. 1991, Kreiss et al. 1987, Takaha et al., 1993, James et al. 1995). DNA sequences of these enzymes are described in the references cited above and in databases, and may be used to clone genes encoding these and further enzymes, for instance using methods described above for α-glucosidases. The activity in the amyloplast of a storage organ may be increased by introduction of one of these genes by any appropriate method of plant transformation, as discussed.

For instance, this may be achieved by the *Agrobacterium-rhizogenes*-based method used by van der Leij et al (1991) for the successful introduction of the *amf* gene product into the amyloplast of potato tubers carrying a mutation at the *amf* locus. The gene used for transformation should preferably be from a species different from that being transformed, to

avoid potential problems of co-suppression. Genes for ß-amylase have been cloned from plants and bacteria (eg. Yoshida et al. 1992, Siggens 1987, Monroe et al. 1991, Kreiss et al. 1987) hence this activity may be introduced into the amyloplast of a storage organ through the use of any of these genes in any appropriate method of plant transformation. The products of these genes are typically targeted to cell compartments other than the plastid in plants, and outside the cell in bacteria. Preparation of a suitable construct for introduction of ß-amylase activity into the amyloplast advantageously may therefore involve selection of a part of the protein that has catalytic activity but lacks targeting sequences. Information about the structure-function relationships of the protein enables a person skilled in the art to select such a region of the protein (eg. Janacek 1994, Kawazu et al. 1987). Construction and introduction of an appropriate plasmid for expression of a ß-amylase in the amyloplast may be achieved using any suitable technique available to the person skilled in the art, as discussed above e.g. for α-glucosidase.

Thus, the present invention relates to increasing the level of MOS in a plant cell in various aspects which are analogous to those disclosed and discussed above in relation to reducing MOS levels.

Activity of a glucan-synthesizing enzyme in a cell or tissue extract or enzyme within a purified

starch granule may be identified by ability of the enzyme, extract or granule to incorporate the glucosyl moiety from ADPG into a glucan via an α-1,4 linkage. The preferred methods of assay are described by Jenner et al. (1994).

Modification of the activity or nature of the granule bound starch synthase activity of a cell may be used to alter the extent or the nature of the change in starch stucture brought about by alteration of MOS content in the cell.

Genes encoding GBSSI from many plant species have been cloned (see Ainsworth et al., 1993), and sequences are available. DNA sequences may be used by a person skilled in the art to clone a GBSSI gene from any suitable species using suitable methods, e.g. as discussed above.

These sequences may be introduced into a plant in such a way as to increase or decrease or alter the nature of the granule-bound starch synthase activity via any suitable transformation technology, again discussed above. Increases may be brought about by introduction of a gene encoding the endogenous enzyme. Decreases may be brought about through introduction of the gene encoding the endogenous enzyme in an antisense orientation. Alteration of the nature of the enzyme may be brought about by the introduction of a gene encoding a GBSSI or a similar protein from another species, or a gene that has been subjected to

mutation such that the properties of the GBSSI it encodes have been modified. A modified GBSSI or a GBSSI from another species may be introduced into a wild-type plant such that the introduced protein is synthesised in addition to the endogenous GBSSI, or it may be introduced into a cell in which endogenous GBSSI activity has been reduced or eliminated through mutation of the expression of antisense RNA.

The present invention will now be illustrated and exemplified with reference to experimental results and the accompanying figures. Further aspects and embodiments of the present invention, and modifications of those disclosed herein, will be apparent to those skilled in the art.

THE FIGURES:

Figure 1a: Elution of solubilised pea starch from a column of Sepharose CL-2B. ■ = absorbance at 595 nm of fractions mixed with iodine. □ = wavelength of maximum absorbance of fractions mixed with iodine

Figure 1b: Elution of ^{14}C from a column of Sepharose CL-2B loaded with the methanol/KCl-insoluble products of incubation of isolated starch granules from pea embryos with ADP[U-^{14}C]glucose. ■ = incubation for 1 h. □ = incubation for 0 h (control). dps: disintegrations per second.

Figure 2: Effect of debranching with isoamylase

on elution profile of 14C from a column of Sepharose CL-2B. Two samples of isolated starch granules from pea embryos were incubated with ADP[U-^{14}C]glucose for 1 h. Methanol/KCl-insoluble products were then incubated with isoamylase (□), or not treated further (■).

Figure 3: Comparison of the incorporation of ^{14}C into methanol/KCl-insoluble products after incubation of starch from wild-type embryos (■) and *lam* mutant embryos (□) with ADP[U-^{14}C]glucose for 1 h.

Figure 4: Comparison of the granule-bound proteins of wild-type and lam mutant pea embryos. Starch granules were boiled in SDS-containing sample buffer and centrifuged, and the supernatant was subjected to SDS-polyacrylamide gel electrophoresis as described by Smith (1990). Tracks 1 and 8 = molecular weight markers (sizes indicated in kDa). Tracks 2-4 = starch from embryos from three different pods of wild-type peas. Tracks 5-7 = starch from embryos from three different pods of *lam* mutant peas. tracks 2-7 all contain protein from the same weight of starch.

Figure 5: Effect of heat-stable soluble compounds from plant extracts on incorporation of ^{14}C from ADP[U-^{14}C]glucose by starch granules. Freshly harvested pea embryos (250-350 mg) were homogenised in a mortar in 100 mM Mops (pH 7.2), 5 mM $MgCl_2$, 50 $ml.l^{-1}$ glycerol, 2 mM DTT, 1 $g.l^{-1}$ bovine serum albumin. The homogenate was either used directly (unwashed starch

granules) or 1-ml aliquots of the homogenate were centrifuged at 10000 g for 5 min to give a supernatant containing soluble material and a pellet containing starch granules. The supernatant was removed and the pellet was washed three times by resuspension in homogenisation medium, followed by centrifugation at 10000 g for 5 min and then resuspended in homogenisation medium to give a final volume of 1 ml. Samples of washed (■) and unwashed (□) starch granules were incubated for 1 h with ADP[U-^{14}C]glucose and the methanol/KCl-insoluble products were subjected to Sepharose CL-2B chromatography.

Figure 6: Effect of α-glucosidase treatment on the stimulatory effect of soluble extract on amylose synthesis. Isolated starch granules and treated and untreated supernatant fractions were those described in Table 2. The products of assays of granules suspended in Mops medium (■), treated supernatant (○) and untreated supernatant (□) were subjected to chromatography on Sepharose CL-2B

Figure 7: Effect of malto-oligosaccharides on incorporation of ^{14}C from ADP[U-^{14}C]glucose into methanol/KCl-insoluble products by starch granules from wild-type pea embryos. Incubations were for 1 h and contained 100 mM maltose (□), 100 mM maltotriose (○) 100 mM maltohexaose (x) or no additions (■).

Figure 8: Comparison of the effects of maltotriose on incorporation of ^{14}C from ADP[U-

^{14}C]glucose by starch granules from wild-type pea embryos (□ = with maltotriose, ■ = without maltotriose) and *lam* mutant pea embryos (○ = with maltotriose, ● = without maltotriose)

Figure 9: Effect of malto-oligosaccharides on incorporation of 14C from ADP[U-^{14}C]glucose into methanol/KCl-insoluble products by starch granules from developing potato tuber. Incubations were for 1 h and contained 100 mM maltotriose (□) or no additions (■).

Figure 10: Elution of debranched, ^{14}C-labelled starch from a column of Sepharose CL-4B. Samples of isolated starch granules from pea embryos and starch granules which had been incubated with ADP[U-^{14}C]glucose for 1 h were incubated with isoamylase prior to loading onto the column. Elution of unlabelled sample, as absorbance at 595 nm of fractions mixed with iodine (■); and elution of ^{14}C-labelled, methanol/KCl-insoluble products from sample incubated with ADP[U-^{14}C]glucose (□).

Figure 11: Effect of pre-treatment with α-glucosidase on the stimulatory influence of soluble extracts of potato tuber on amylose synthesis. Two, replicate 5-g samples of developing tuber were prepared. One was used for isolation of starch granules as described in Experimental procedures. The other was extracted in a mortar in a total of 10 ml of 50 mM sodium acetate (pH 5.5) with 0.5 g

polyvinylpolypyrrolidone. After centrifugation, the supernatant (soluble extract) was boiled for 5 min then samples were incubated for 18 h with or without 1.25 unit ml^{-1} α-glucosidase (yeast) at 35°C. After boiling again, all samples were brought to pH 7. The starch granules were resuspended at 25 mg ml^{-1} in Medium A (■), α-glucosidase-treated soluble extract (○) or untreated soluble extract (□) and incubated for 1 h with ADP[U-^{14}C]glucose. The methanol/KCl-insoluble products were subjected to chromatography on Sepharose CL-2B.

Figure 12: Comparison of the effects of maltotriose on incorporation of ^{14}C from ADP[U-^{14}C]glucose by starch granules from wild-type and *lam* mutant pea embryos. Wild-type: □, with 100 mM maltotriose; ■, without maltotriose *lam* mutant : ○, with 100 mM maltotriose; ●, without maltotriose

Figure 13: Effect of malto-oligosaccharides on incorporation of ^{14}C from ADP[U-^{14}C]glucose into methanol/KCl-insoluble products by starch granules from developing potato tuber. Incubations contained 100 mM maltotriose (□) or no additions (■).

All documents mentioned herein are incorporated by reference.

MATERIALS AND METHODS

Plant material

Peas were of a round-seeded line (*Pisum sativum* L., BC1/12 RR: see Smith 1990 for derivation of this line) or a low-amylose (*lam*) mutant line (SIM 503) derived from the round seeded line (Denyer at al. 1995a). Both were obtained from Dr. Cliff Hedley and Dr. Trevor Wang, John Innes Centre, Norwich. Plants were grown as described by Denyer et al. (1995a). Potatoes (*Solanum tuberosum* L. cv Desiree) were grown as described by Edwards et al. (1995).

Preparation of starch

Starch was prepared from developing pea embryos essentially according to Smith (1990). Approximately 1-3 g of embryos of fresh weight 200-400 mg was homogenised in a mortar with 5-15 ml 100 mM Tris-acetate (pH 7), 0.5 M NaCl, 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA) at 4°C. The homogenate was filtered through two layers of Miracloth (Behring Diagnostics, La Jolla, CA, USA), and the residue was washed in the filter cloth with a further 5-15 ml of extraction medium. The combined filtrate was stirred at 4°C for 30 min and then subjected to centrifugation at 2000 *g* for 10 min. The supernatant and the green material from the surface of the pellet were discarded and the pellet was resuspended in 10 ml extraction medium. This centrifugation and resuspension procedure was repeated, once with extraction medium and three times

with 50 mM Tris-acetate (pH 8), 1 mM DTT, 1 mM EDTA. The pellet was resuspended in acetone cooled to -20°C and allowed to settle for 15 min, and then the supernatant was discarded. This acetone wash was repeated twice more and the washed starch was dried under air at room temperature. The starch was either used immediately or stored at -20°C for up to 2 months.

Starch was prepared from developing potato tubers (each 10-100 g fresh weight) essentially as described above for pea embryos except that the extraction medium was 50 mM Tris-acetate (pH 8), 1 mM DTT, 1 mM EDTA, stirring at 4°C was omitted and the total number of washes in extraction medium was 3-4.

Incubation of granules with ADPglucose

Starch granules were resuspended at 1mg per 40 µl in 100 mM 3-(N-morpholino)propanesulphonic acid (Mops, pH 7.2), 5 mM $MgCl_2$, 50 $ml.l^{-1}$ glycerol, 2 mM DTT, 1 $g.l^{-1}$ bovine serum albumin. Each incubation contained 10 µl starch suspension and 90 µl 100 mM Bicine (pH 8.5), 25 mM potassium acetate, 10 mM DTT, 5 mM EDTA, 1 mM ADP[U-^{14}C]glucose (Amersham International, Bucks, UK) at 18.5 $GBq.mol^{-1}$ and was incubated at 25°C for 1 hour. The incubation was terminated either by heating for 2 min at 90°C followed by the addition of 3 ml 750 $ml.l^{-1}$ aqueous methanol containing 10 $g.l^{-1}$ KCl (methanol-KCl) or by the addition of methanol-KCl

alone. After incubation at room temperature for a minimum of 5 min, the precipitated starch was collected by centrifugation at 2000 *g* for 5 min. The supernatant was discarded and the pellet was resuspended in 0.3 ml distilled water. This methanol-KCl wash, centrifugation and resuspension was repeated twice more except that after the final centrifugation the distilled water was omitted and the pellet was dried in air at room temperature for a minimum of 10 min. The washed starch pellet was dissolved in 50 µl of 1 M NaOH, diluted with 50 µl distilled water, and subjected to gel filtration chromatography.

Isoamylase digestion

Starch granules were incubated with ADPglucose and washed with methanol-KCl as described above. The dried starch pellet was resuspended in 25 µl 50 mM sodium acetate (pH 3.5) containing 4000 units isoamylase (Sigma, Poole, Dorset, UK) and incubated at 37°C for 24 h. The starch was then dissolved in 50 µl 1 M NaOH, diluted with 25 µl distilled water, and subjected to gel filtration chromatography.

Gel filtration chromatography on Sepharose CL-2B

Samples were either unlabelled starch which was dissolved in 1 M NaOH at 20 mg. ml^{-1} and diluted with distilled water to 0.5 M NaOH or ^{14}C-labelled starch prepared as described above. A 50-µl sample of

dissolved starch was applied to a column (0.5 cm i.d. x 30 cm) of Sepharose CL-2B equilibrated with 100 mM NaOH, and eluted with this solution (Denyer et al. 1995a). Fractions of 0.15-0.17 ml were collected at the rate of one fraction per minute. Glucans in fractions from unlabelled samples were detected by their reaction with iodine as described in Denyer et al. (1995a). Radioactivity in fractions from samples containing labelled glucans was determined by liquid scintillation counting on a Wallac 1410 counter (Wallac UK, Milton Keynes, UK) using 1 or 3 ml HiSafe II liquid scintillation fluid (Beckman Ltd., High Wycombe, Bucks, UK) per fraction.

SDS-polyacrylamide gels

As described in Denyer et al. (1995).

Assay of granule-bound starch synthase activity

Assays contained 100 mM Bicine (pH 8.5), 25 mM potassium acetate, 10 mM DTT, 5 mM EDTA, 1 mM ADP[U-^{14}C]glucose (Amersham International, Bucks, UK) at 2.3 GBq mol^{-1} and 10 µl of a suspension of starch granules in a final volume of 100 µl. Assays were incubated at 25°C for 15 min and then heated to 90°C for 2 min. Controls were heated to 90°C immediately after the addition of starch granules to the assay. All assays and controls were duplicated. Starch was precipitated with methanol-KCl and washed as described above

(Incubation of starch granules with ADPglucose) except that the final pellets were dissolved in 0.3 ml of distilled water followed by 3 ml HiSafe II liquid scintillation fluid. Radioactivity was determined by liquid scintillation counting.

RESULTS

Identification of the products of GBSS

The starch synthase activity of starch granules isolated from pea embryos was measured as the incorporation of ^{14}C from ADP[U-^{14}C]glucose into material which was insoluble in 750ml.l^{-1} methanol containing 10g.l^{-1} KCl (methanol/KCl). This activity was specific for ADPglucose (there was almost no activity with UDPglucose - at 1mM the activity was less than 3% of that with ADPglucose at the same concentration) and the incorporation was linear with respect to time for at least 1h. The ^{14}C-labelled methanol/KCl-insoluble material was completely solubilised after incubation with amyloglucosidase (not shown), showing that the products of the reaction were glucans (α-1,4, α-1,6 linked). In control assays, which were terminated immediately after the addition of the substrate, incorporation of ^{14}C into glucans was minimal (less than 3% of that in assays which were incubated for 1h).

Sepharose CL-2B chromatography of pea starch results in a clear separation of the amylose and

amylopectin components (Fig. 1a). Amylopectin is the first peak of glucan to elute, and amylose the second (Denyer et al. 1995a). When the methanol/KCl-insoluble products from incubation of isolated starch granules with ADP[U-^{14}C]glucose were subjected to chromatography on a column of Sepharose CL-2B, ^{14}C eluted predominantly in the amylopectin peak (Fig. 1b). In seven experiments, the average recovery of ^{14}C in the amylopectin peak as a percentage of the total ^{14}C eluted from the column was 78 ± 12% (mean ± SD). The total ^{14}C recovered in the fractions eluting from the column in these and subsequent experiments was 70-100% of that applied to the column, hence there were no major losses of labelled products on the column.

To investigate whether the ^{14}C co-eluting with amylopectin was in amylopectin or in high-molecular-weight amylose molecules, the products were treated with isoamylase to debranch glucans prior to application to the column. After isoamylase treatment the ^{14}C eluted much later from the column (Fig. 2), indicating a lower molecular weight. In control treatments with buffer instead of isoamylase, the position of elution of ^{14}C was unaltered (data not shown). These results are consistent with incorporation of ^{14}C glucose via GBSS into the branches of amylopectin molecules rather than into linear polymers.

The starch granules used in these experiments

were prepared by washing insoluble extracts of developing pea embryos several times in aqueous media, then in cold acetone followed by drying in air and storage at -20°C for up to two months before being assayed. To determine whether these preparation or storage methods influenced the type of products made by the GBSSs, granules prepared by the following alternative methods were assayed: 1) freshly-prepared granules which had not been stored at -20°C and 2) freshly-prepared granules which were washed with aqueous media but not washed with acetone or air-dried. In both of the above experiments the elution profile of ^{14}C was essentially the same as that shown in Fig 1b: the incorporation was very largely into amylopectin.

Hylton et al. (1995) found that mechanically-damaged starch granules from barley endosperm had a higher GBSS activity than intact granules, and that most of the increase in activity was due to isoforms other than GBSSI. In the light of this result we investigated whether in our experiments the pattern of incorporation of glucose was affected by damage to the granules. Granules were prepared with the minimum of mechanical damage by chopping the developing pea embryos with a razor blade into extraction medium rather than by grinding in a mortar. The activity (per mg starch) of granules prepared from chopped pea embryos was approximately half that of granules

prepared by grinding. However, there was no change in the nature of the products which were synthesised (not shown). There was no change in the pattern of incorporation of ^{14}C into polymers: most of the incorporation in the granules prepared by chopping rather than grinding was into amylopectin (not shown).

Analysis of the size of the ^{14}C-labelled amylopectin chains

To investigate the average length of amylopectin chains into which ^{14}C was incorporated, the products of the incubation of pea starch granules with ADP[U-^{14}C]glucose were debranched with isoamylase and subjected to chromatography on a column of Sepharose CL-4B. The first peak of glucan to elute from the column in the experiment shown in Figure 10 consisted of amylopectin which had not been completely debranched. Glucan eluting later from the column consisted of chains of debranched amylopectin. Comparison of the elution profiles of ^{14}C-labelled and unlabelled amylopectin chains showed that the peak of the former eluted earlier than the latter. This suggests that the chains elongated via starch synthases during the incubation were considerably longer than the average chains in the amylopectin molecules.

Comparison of starch from wild-type and *lam* mutant

embryos

To investigate whether the observed elongation of amylopectin was due to the activity of GBSSI, GBSSII or both of these isoforms, we compared the incorporation of ^{14}C-glucose from ADP[U-^{14}C]glucose into starch from wild-type and *lam* mutant pea embryos. Starch granules from *lam* mutant embryos lack GBSSI, therefore incorporation into these granules must be via GBSSII alone. The incorporation into granules from *lam* mutant embryos was entirely into the amylopectin fraction (Figure 3). This shows that GBSSII of pea embryos can elongate amylopectin branches within the granule.

As reported previously, the total incorporation of ^{14}C glucose into granules of *lam* mutant embryos was lower than granules of wild-type embryos on a starch basis (Denyer et al. 1995a). To discover whether this reduction in incorporation was due solely to the absence of GBSSI, or whether the mutation also resulted in a reduction in the amount of GBSSII, granule-bound proteins from both starches were extracted and compared on SDS-polyacrylamide gels (Fig 4). The amount of GBSSII protein per mg of starch was the same in the wild-type and the mutant. Assuming that the activities of the GBSSs were not grossly affected by structural differences between the starches, this suggests that the difference between the starches of wild-type and *lam* embryos in their

ability to incorporate glucose from ADPglucose into amylopectin is attributable to the activity of GBSSI.

The effect of the soluble fraction of storage organs on the products of the GBSSs

The above experiments show that GBSSI in isolated starch granules transfers glucose from ADPglucose largely to amylopectin rather than to amylose molecules. Since this isoform is responsible for the synthesis of amylose *in vivo*, some factor(s) present *in vivo* but not in isolated starch granules may be required for amylose synthesis. To investigate whether soluble factors required for amylose synthesis had been washed out of the starch granules during their preparation, we compared the methanol/KCl-insoluble products of incorporation from ADP[U-^{14}C]glucose by crude homogenates of developing pea embryos (containing both soluble and insoluble fractions) and washed starch granules derived from the homogenates (Figure 5). Crude homogenates gave a higher total incorporation of ^{14}C-glucose than washed starch granules, due to an appreciably greater incorporation into amylose. When the soluble fraction derived by centrifugation of crude extracts was added back to the washed starch granules, a stimulation of incorporation into amylose was observed equivalent to the amylose synthesis in crude homogenates. Boiling the soluble extract prior to this addition did not

effect its ability to stimulate amylose synthesis, shwoing that the stimulatory factor was heat-stable and suggesting that soluble enzymes were not required (not shown).

We investigated whether developing potato tubers also contained soluble factors which could stimulate the activity of GBSS. GBSS activity in crude homogenates of tuber is considerably greater than that in washed starch granules derived from the same homogenate. As with pea embryos, the addition of boiled, soluble extract to the washed starch granules stimulates the GBSS activity (Table 1) and promotes the synthesis of amylose (Figure 11).

Taken as a whole, these experiments suggest that soluble extracts of pea embryos and potato tubers contain non-enzymic factors which stimulated amylose synthesis in the granule. Leloir et al. (1961) found that GBSS activity was stimulated by the presence of maltose, maltotriose or maltotetraose and presented evidence to suggest that these malto-oligosaccharides could act as substrates for GBSSs in granules isolated from *Phaseolus vulgaris*. Although the work of Leloir et al. involved the transfer of glucose from UDPglucose, an activity which is not present to any significant extent in our preparations of starch granules, it seemed possible that the stimulatory factors in our soluble extracts are malto-oligosaccharides which act as primers for amylose

synthesis. To investigate this, boiled, soluble extracts of potato tuber were treated with α-glucosidase (maltase) to remove malto-oligosaccharides. This treatment eliminated the ability of the extracts to stimulate GBSS activity of isolated starch granules, and to promote incorporation of ^{14}C into amylose (Table 2, Figure 6).

In three separate experiments, the granule-bound starch synthase activity relative to that of starch granules resuspended in Medium A was 154.3% ± 16.5% (mean ± SD) for starch granules resuspended in untreated soluble extract and 109.8% ± 8.9% (mean ± SD) for starch granules resuspended in α-glucosidase-treated soluble extract. The results from the analysis of one of these experiments is shown in Figure 11. Taken as a whole, these experiments suggest that soluble extracts of pea embryos and potato tubers contain malto-oligosaccharides which can stimulate amylose synthesis in starch granules.

We therefore investigated whether purified malto-oligosaccharides could promote amylose synthesis by isolated granules. The results are presented below.

The effects of malto-oligosaccharides on GBSS activity

When supplied to isolated starch granules from pea embryos, a range of small malto-oligosaccharides, from maltose to maltohexaose, stimulated the incorporation of ^{14}C-glucose from ADP[U-^{14}C]glucose

into material eluting from Sepharose CL-2B in the same position as, or slightly after amylose. Incorporation into amylopectin was reduced (Figure 7), and incorporation into material eluting from a Sepharose CL-2B column in the same position as, or slightly after, amylose was greatly stimulated. Maltoheptaose and a mixture of malto-oligosaccharides with from four to ten glucose units had a similar effect (data not shown). With maltose, the effect was apparent at concentrations of 10mM, and was fully saturated at 100mM. Neither 100mM glucose nor 100mM cellobiose had any effect on incorporation, showing that the stimulatory effect was specific for malto-oligosaccharides (data not shown).

To determine whether the synthesis of amylose in the presence of malto-oligosaccharides was due to the activity of GBSSI, GBSSII or both, the products formed in the presence of maltotriose by granules from wildtype and *lam* mutant embryos were compared (Figure 8 and Figure 12). Maltotriose had no effect on the products synthesised by starch granules from *lam* mutant embryos, showing that GBSSII alone could not synthesise amylose in the presence of malto-oligosaccharides. The amylose synthesis observed in wild-type granules in the presence of malto-oligosaccharides is therefore likely to be due to the activity of GBSSI.

The effect of malto-oligosaccharides on the GBSS activity of starch from potato tubers

We compared the effects of malto-oligosaccharides on pea starch with the effects on starch isolated from developing potato tubers. The Sepharose CL-2B column was less effective in separating amylose and amylopectin from potato starch than from pea starch (not shown) but it was nonetheless clear that in the absence of maltotriose, incorporation of ^{14}C from ADP[U-^{14}C]glucose was largely into the amylopectin peak. See Figure 13. In the presence of maltotriose, the incorporation into amylopectin was reduced dramatically and there was an increase in incorporation into amylose (Figure 9). These results are similar to those obtained with pea starch and were consistent with the idea that GBSSI of potato starch elongates amylopectin in the absence of maltotriose and synthesises amylose in the presence of maltotriose.

DISCUSSION

Our results show that both GBSSI and GBSSII of developing pea embryos elongate glucans within starch granules. GBSSI can elongate the branches of amylopectin as well as elongating maltose and higher oligomers of glucose. GBSSII can elongate amylopectin but apparently cannot elongate short malto-oligosaccharides. Thus only GBSSI can initiate the

synthesis of amylose. We cannot rule out from our present studies the possibility that GBSSII could contribute to the elongation of amylose, once these molecules have reached a certain size.

The properties of the GBSSI of developing pea embryo described in this paper should represent the properties of this class of starch synthases generally. Baba et al. (1987) showed that granules isolated from sweet potato tubers incorporated ^{14}C from ADP[U-^{14}C]glucose primarily into amylopectin. Malto-oligosaccharides were not supplied in these experiments. Leloir et al. (1961) showed that malto-oligosaccharides could act as primers for starch synthesis in granules from developing beans. We have shown that malto-oligosaccharides are required to stimulate amylose synthesis in isolated granules from potato tubers as well as pea embryos.

Starches from many starch storing organs possess GBSSs very similar in size, antigenic properties and/or sequence to GBSSII of pea. The properties of all members of the GBSSII class are likely to be similar, so elongation of amylopectin via these isoforms within the granules of starches other than pea may also occur. However, there is variation between starches from different sources in the types and numbers of GBSSs other than GBSSI (Smith et al. 1995). At present, we have no information on the ability of GBSSs other than GBSSI and GBSSII to

elongate amylopectin within the granule. Starches from different sources also vary in the extent to which GBSSs other than GBSSI contribute to the total GBSS activity. For example the activity of GBSSII is very low compared to the activity of GBSSI in potato tubers (Edwards et al. 1995). This may account for the much lower incorporation into amylopectin in the presence of maltotriose in potato starch than in pea starch (Figs 8 and 9). Thus the contribution of GBSSs to amylopectin synthesis within the granule may differ between starches because both the total activity and the properties of these enzymes may vary.

In isolated granules, the overall activity of GBSSI and the nature of its products depends upon the availability of malto-oligosaccharides. We have demonstrated the presence in crude extracts of pea and potato of compounds which are susceptible to degradation by α-glucosidase, and which stimulate amylose synthesis in isolated granules. These malto-oligosaccharides may be produced *in vivo* by the action of starch-degrading enzymes. It has long been known that such degradative enzymes are present during starch synthesis and some of these have been shown to be plastidial. Our results suggest that one role of the degradative enzymes may be to produce primers for amylose synthesis.

TABLE 1

	GBSS activity (μmol.min^{-1}.g^{-1} FWT)
Homogenate	0.171, 0.198
Washed pellet	0.063, 0.093
Washed pellet + boiled supernatant	0.149, 0.147

GBSS activity in fractions of crude extracts of developing potato tubers.

Samples of developing tubers were homogenised, and washed pellet (containing starch granules) and supernatant (containing soluble material) fractions were prepared as described in Figure 5 for pea embryos. Starch synthase activity was assayed in the homogenate and the supernatant, and the latter value subtracted from the former to give GBSS activity in the homogenate. Half of the washed pellet was resuspended in 1.5 ml extraction medium and half in 1.5ml boiled supernatant, and both samples were assayed for starch synthase activity. Boiled supernatant contained no detectable starch synthase activity. In these experiments, amylopectin (potato; Sigma, Poole, Dorset, UK) was included in the assays at 5 mg ml^{-1}. Values are for samples from two, separately-extracted tubers.

TABLE 2

	GBSS activity (nmol.min^{-1}.mg^{-1} starch)
buffer	0.708
untreated supernatant	1.170
treated supernatant	0.751

Effect of α-glucosidase treatment on stimulation of GBSS activity by crude extracts of developing potato tubers.

Two, replicate samples, each of 5 g, of developing tuber were prepared. One was used for isolation of acetone-washed starch granules as described in Materials and Methods. The other was extracted in a mortar in a total of 10 ml of 50 mM Na acetate (pH 5.5) with 0.5 g polyvinylpolypyrrolidone. After centrifugation, the supernatant was boiled for 5 min then samples were incubated overnight with or without 1.25 unit.ml^{-1} α-glucosidase (yeast) at 35°C. After boiling again, all supernatant samples were brought to pH 7 and used to resuspend starch granules (prepared from the other sample of tuber) for assay. Buffer = starch resuspended in Mops medium as described in Materials and Methods, untreated supernatant = supernatant incubated without α-glucosidase, treated supernatant = supernatant incubated with α-glucosidase.

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CLAIMS

1. A method of producing α-glucan in a cell having α-glucan synthase activity, the method including modulating the malto-oligosaccharide (MOS) content in the cell.

2. A method according to claim 1 wherein the α-glucan synthase activity is Granule Bound Starch Synthase I (GBSSI) activity.

3. A method according to claim 1 or claim 2 wherein the production of the α-glucan by the α-glucan synthase activity is modified by the change in MOS content.

4. A method according to claim 3 wherein the cell has an α-glucan polymer storage compartment and the method includes modulating the MOS content in the α-glucan polymer storage compartment.

5. A method according to claim 4 wherein the α-glucan polymer storage compartment is an amyloplast.

6. A method according to any one of the preceding claims including modulating the MOS content such that the α-glucan produced has a modulated content of unbranched and/or branched α-glucan.

7. A method according to claim 6 including modulating the MOS content such that the α-glucan produced has reduced levels of unbranched α-glucan polymer.

8. A method according to claim 6 including modulating the MOS content such that the α-glucan produced has a branched α-glucan content of different nature from that of an equivalent cell in which the MOS content is not modulated.

9. A method according to claim 7 or claim 8 wherein the MOS content is modulated using an enzyme that uses MOS as a substrate.

10. A method according to claim 9 wherein the level of MOS is reduced.

11. A method according to claim 9 or claim 10 characterised in that the enzyme is not GBSSI.

12. A method according to claim 9 or claim 10 wherein the enzyme is an α-glucosidase (EC 3.2.1.20) having a relatively strong maltase activity (i.e. low K_m for maltose).

13. A method according to claim 9 or claim 10 wherein the enzyme is a maltase of *Bacillus amylolyticus* or

the maltase encoded by the *MAL6* locus of *Saccharromyces carlsbergensis* or a mutant, derivative, variant or allele thereof.

14. A method according to any one of claims 6 to 8 wherein the level of MOS is increased.

15. A method according to claim 14 wherein the level of MOS is increased by an enzyme that generates MOS in the cell.

16. A method according to claim 15 wherein the enzyme is a β-amylase, an α-amylase, a disproportionating enzyme, a debranching enzyme or a starch phosphorylase.

17. A method according to claim 15 or claim 16 wherein the enzyme has low or negligible activity against intact starch granules.

18. A method according to any one of claims 9 to 13 including incorporating nucleic acid encoding for the production of the enzyme into the cell.

19. A method according to any one of claims 15 to 17 including incorporating nucleic acid encoding for the production of the enzyme into the cell.

20. A method according to any preceding claim wherein the produced α-glucan is removed from the cell.

21. A method according to claim 20 wherein the α-glucan is purified.

22. A method according to claim 20 or claim 21 wherein the α-glucan is formulated into a composition.

23. A method according to any preceding claim wherein the α-glucan is starch.

24. A method according to any preceding claim wherein the unbranched α-glucan is amylose.

25. A method according to any preceding claim wherein the branched α-glucan is amylopectin.

26. An α-glucan producing cell having glucan synthase activity wherein the MOS content in the cell is modulated and the α-glucan content is altered as compared to an equivalent cell having wild-type MOS level.

27. A cell according to claim 26 which includes a heterologous nucleic acid of sequence which expresses an enzyme which uses MOS as a substrate.

28. A cell according to claim 26 which includes a heterologous nucleic acid of sequence which expresses an enzyme which generates MOS.

29. A cell according to any one of claims 26 to 28 which is comprised in a plant, a plant part or a plant propagule.

30. A method of producing a cell according to claim 27 or claim 28, the method including incorporating a heterologous nucleic acid having a sequence encoding for a MOS modulating enzyme into the cell.

31. A method according to claim 30 which includes recombining the heterologous nucleic acid with the cell genome nucleic acid such that it is stably incorporated therein.

32. A method according to claim 30 or claim 31 wherein the nucleic acid is introduced in the form of a vector.

33. A method according to any of claims 30 to 32 wherein the α-glucan is starch

34. A method according to any of claims 30 to 33 wherein the unbranched α-glucan is amylose.

35. A method according to any of claims 30 to 34 wherein

the branched α-glucan is amylopectin.

36. A plant including a cell according to any one of claims 26 to 28.

37. A plant which is a sexually or asexually propagated off-spring, clone or descendant of a plant according to claim 36, or any part or propagule of said plant, off-spring, clone or descendant.

38. A part or propagule of a plant according to claim 36.

39. A method of producing a plant including α-glucan producing cells having glucan synthase activity wherein the MOS content in the cell is modulated and the α-glucan content is altered as compared to an equivalent cell having wild-type MOS level, the method including incorporating a heterologous nucleic acid having a sequence encoding for a MOS modulating enzyme into a plant cell and regenerating a plant from said plant cell.

40. A method according to claim 39 wherein said heterologous nucleic acid when in cells of the plant expresses an enzyme which uses MOS as a substrate.

41. A method according to claim 39 wherein said heterologous nucleic acid when in cells of the plant

expresses an enzyme which generates MOS.

42. A method according to any one of claims 39 to 41 including sexually or asexually propagating or growing off-spring or a descendant of said plant.

43. A method of obtaining α-glucan, the method including removing α-glucan from a cell according to any of claims 26 to 29.

44. A method of obtaining α-glucan, the method including removing α-glucan from a plant, plant part or plant propagule according to any of claims 36 to 38.

45. A method according to claim 43 or claim 44 including purifying the α-glucan.

46. A method according to any of claims 43 to 45 including formulating the removed α-glucan into a composition.

47. A method according to any of claims 43 to 46 wherein the removed α-glucan is starch.

48. α-glucan produced by processing a cell, plant, or part or propagule of a plant provided or as claimed in any of claims 1 to 42.

49. α-glucan according to claim 48 which is starch.

50. A transformed cell having modified carbohydrate composition obtainable by causing same cell to express

(a) a suitable enzyme capable of modulating MOS content and

(b) a suitable enzyme capable of carbohydrate polymer synthesis,

where at least one of said enzymes is exogenous to same cell.

51. A transformed cell according to claim 50 wherein said enzyme (a) is capable of using MOS as a substrate.

52. A transformed cell according to claim 50 or claim 51 wherein said enzyme (b) has α-glucan synthase activity.

53. A transformed cell according to claim 52 wherein the α-glucan synthase activity is capable of modifying branched α-glucan polymers.

54. A transformed cell according to claim 53 wherein the α-glucan synthase activity is GBSSI activity

55. A transformed cell according to any of claims 50 to 54 wherein the enzyme capable of modulating the MOS content or using MOS as a substrate is selected from:

(a) an α-glucosidase (EC 3.2.1.20) having a relatively strong maltase activity (i.e. low K_m for maltose);

(b) a maltase of *Bacillus amylolyticus* or a mutant,

derivative, variant or allele thereof;

(c) a maltase encoded by the *MAL6* locus of *Saccharromyces carlsbergensis* or a mutant, derivative, variant or allele thereof;

(d) a β-amylase with low or negligible activity against intact α-glucan granules;

(e) an α-amylase with low or negligible activity against intact α-glucan granules;

(f) a disproportionating enzyme with low or negligible activity against intact α-glucan granules;

(g) a debranching enzyme with low or negligible activity against intact α-glucan granules; and

(h) a starch phosphorylase with low or negligible activity against intact α-glucan granules.

56. A transformed cell with modulated MOS content.

57. A cell according to any of claims 50 to 56 which is a plant cell.

58. A cell according to claim 57 which is a cereal endosperm, cassava tuber, potato tuber or pea embryo cell.

59. A cell according to claim 57 or claim 58 which is comprised in a plant.

60. A plant including a cell according to claim 57 or claim 58.

61. A plant which is a sexually or asexually propagated off-spring, clone or descendant of a plant according to claim 60, or any part or propagule of said plant, off-spring, clone or descendant.

62. A part or propagule of a plant according to claim 60.

63. A method of producing carbohydrate in a transformed cell, the method including modulating the amount of MOS in a transformed cell which contains an enzyme activity capable of carbohydrate polymer synthesis, and subsequent extraction of said carbohydrate from the transformed cell.

64. A method of producing carbohydrate in a transformed cell, the method including modulating the amount of MOS in a cell by expressing a suitable glucoamylase activity in a cell which contains a starch synthase activity capable of modifying amylose and/or amylopectin, and subsequent extraction of said carbohydrate from the transformed cell.

65. A method of producing carbohydrate in a transformed cell, the method including modulating the amount of MOS in a cell by expressing a suitable α-glucosidase (EC 3.2.1.20) activity in a cell which contains a starch synthase activity capable of modifying amylose and/or amylopectin, and subsequent extraction of said carbohydrate from the transformed cell.

66. A method according to any of claims 63 to 65 wherein the transformed cell is a plant cell.

67. A method according to claim 66 wherein the plant cell is comprised in a plant.

68. A method according to any of claims 63 to 66 which comprises, subsequent to said extraction, formulation of said carbohydrate into a composition.

Figure 1(a)

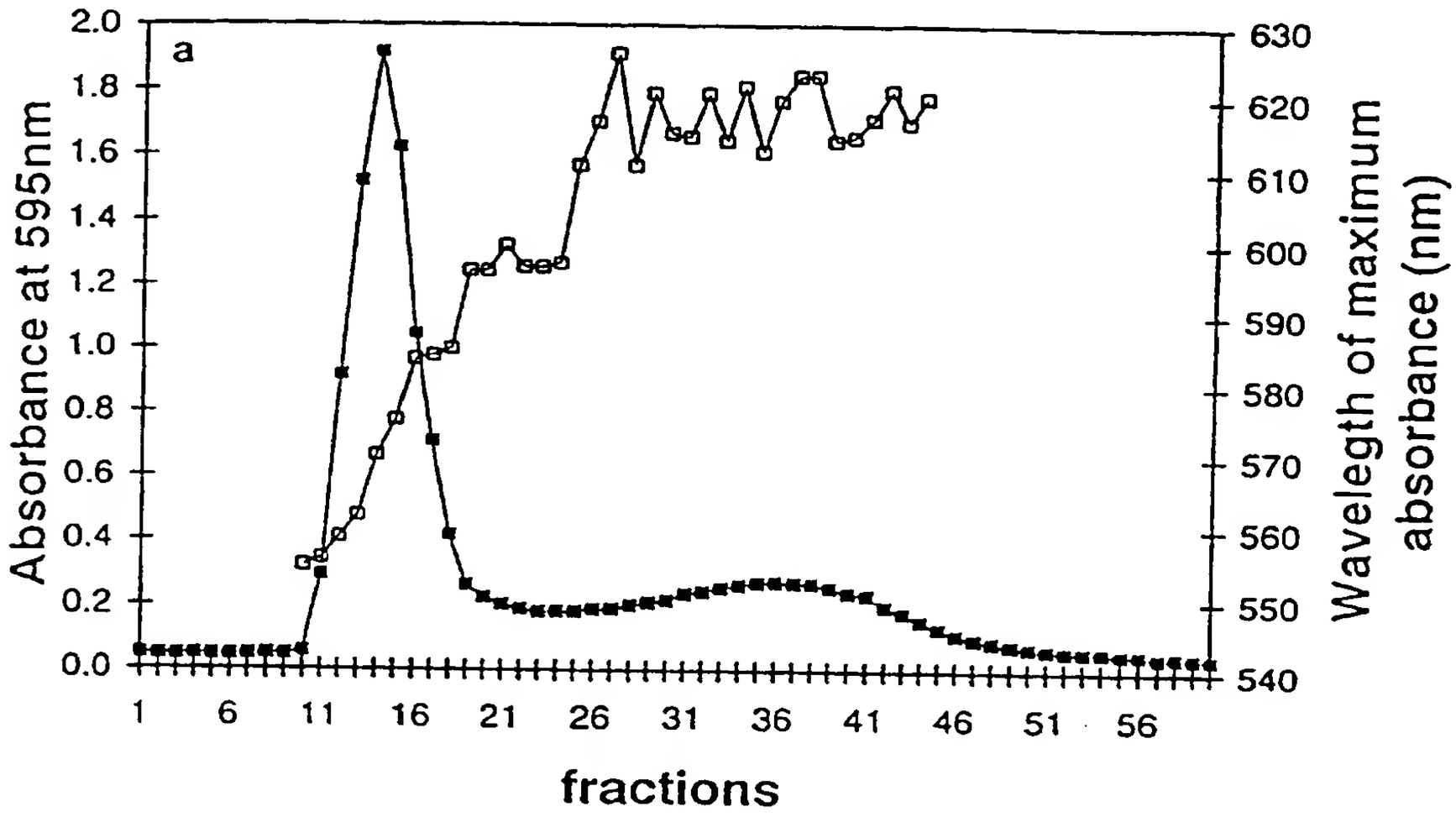
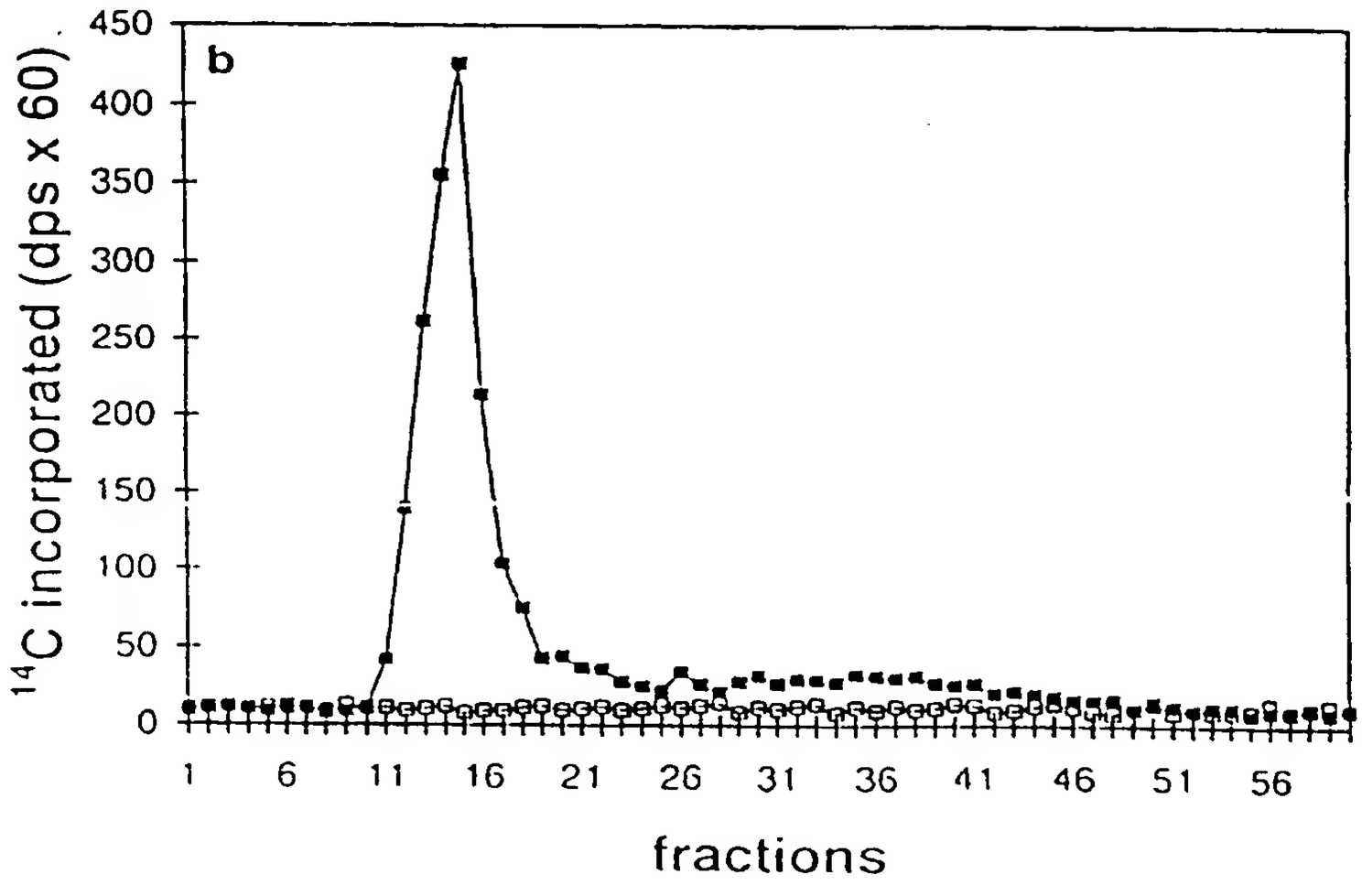
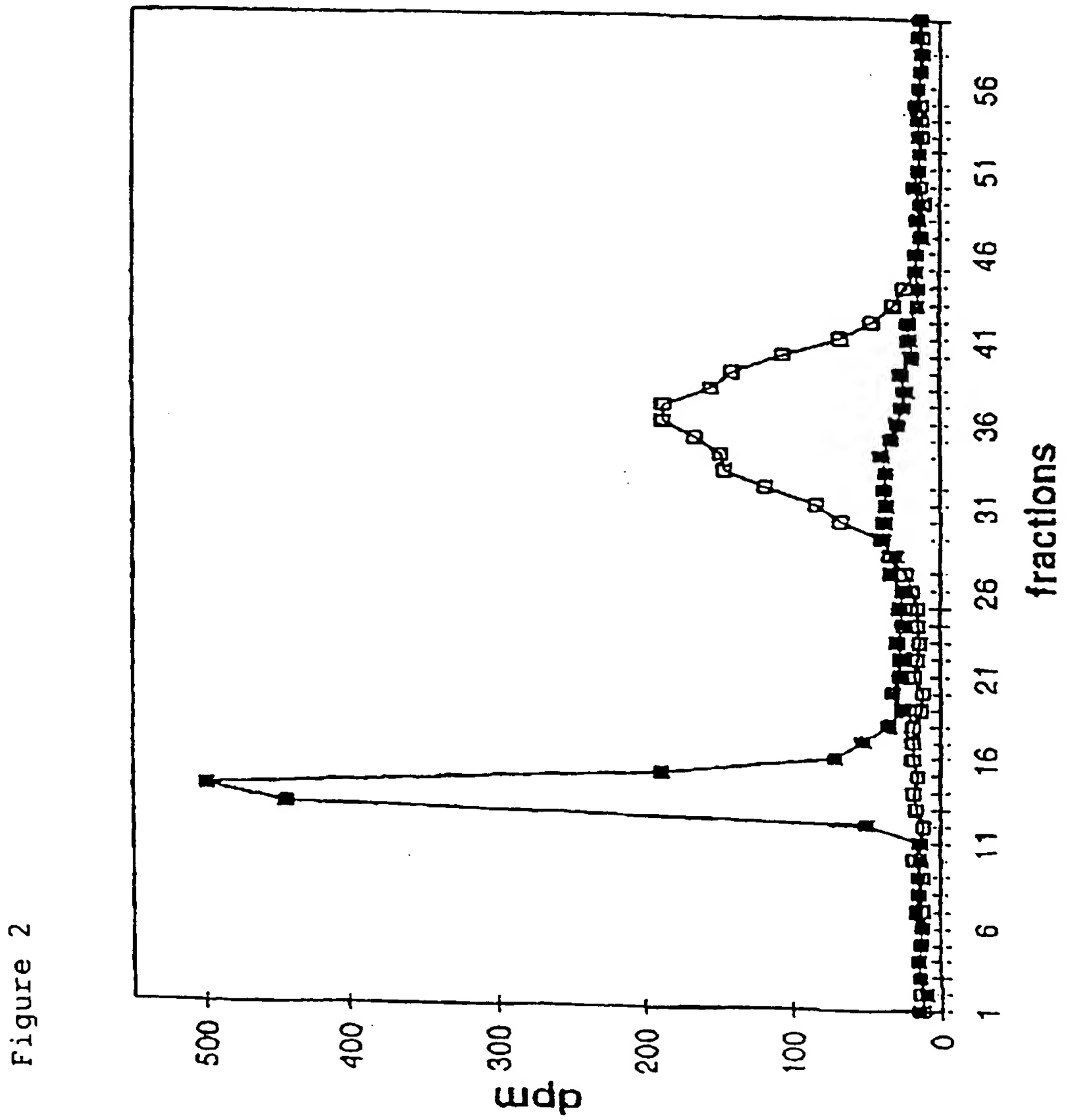


Figure 1(b)

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fractions

Figure 2

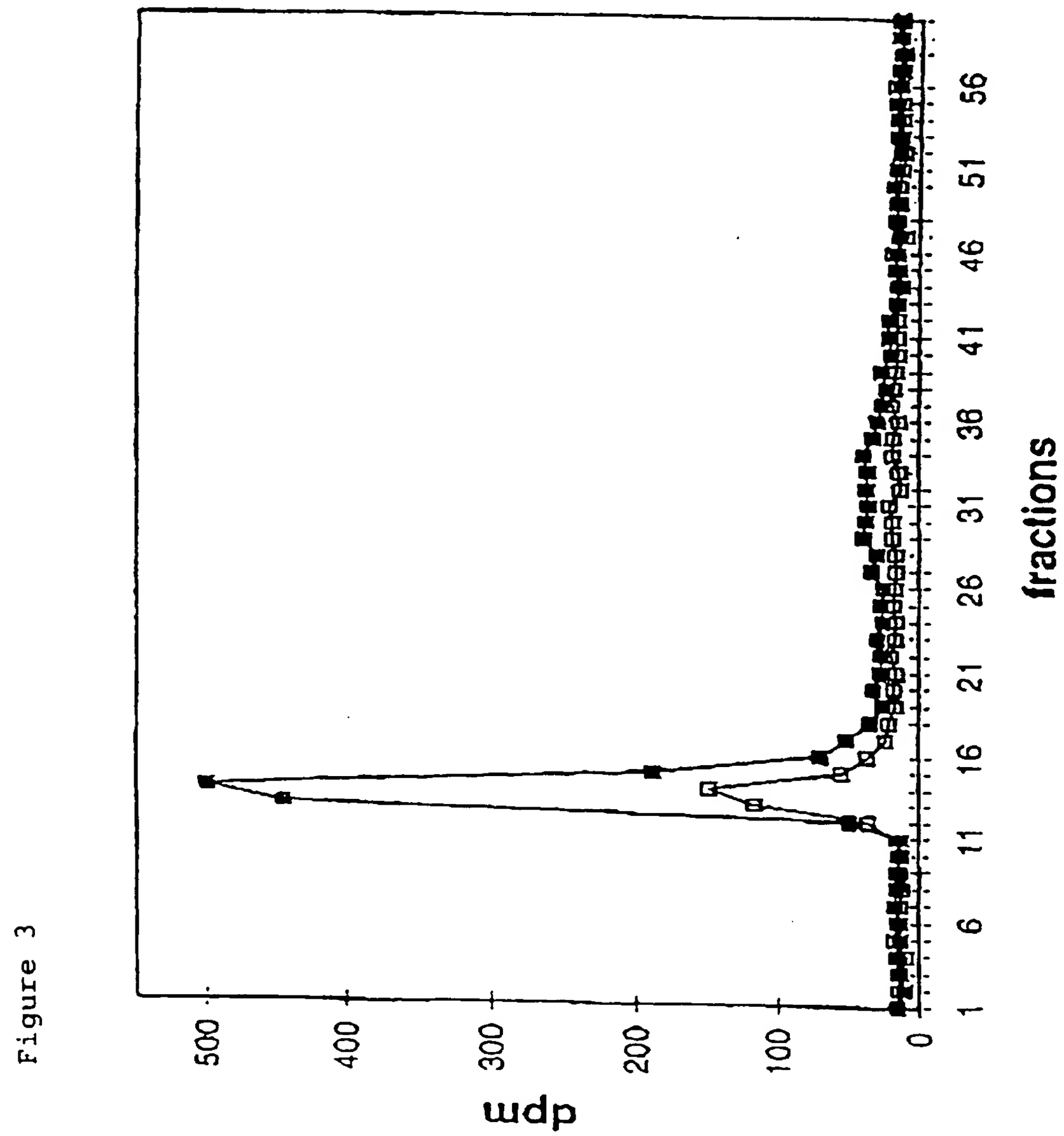
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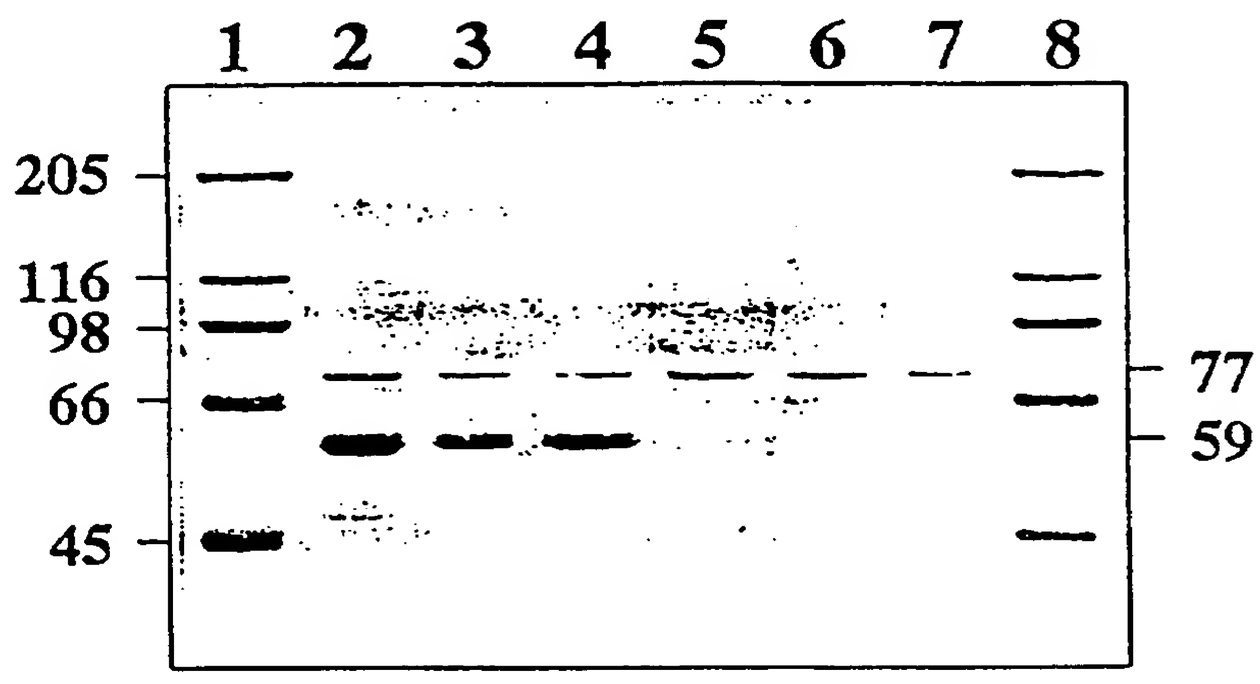
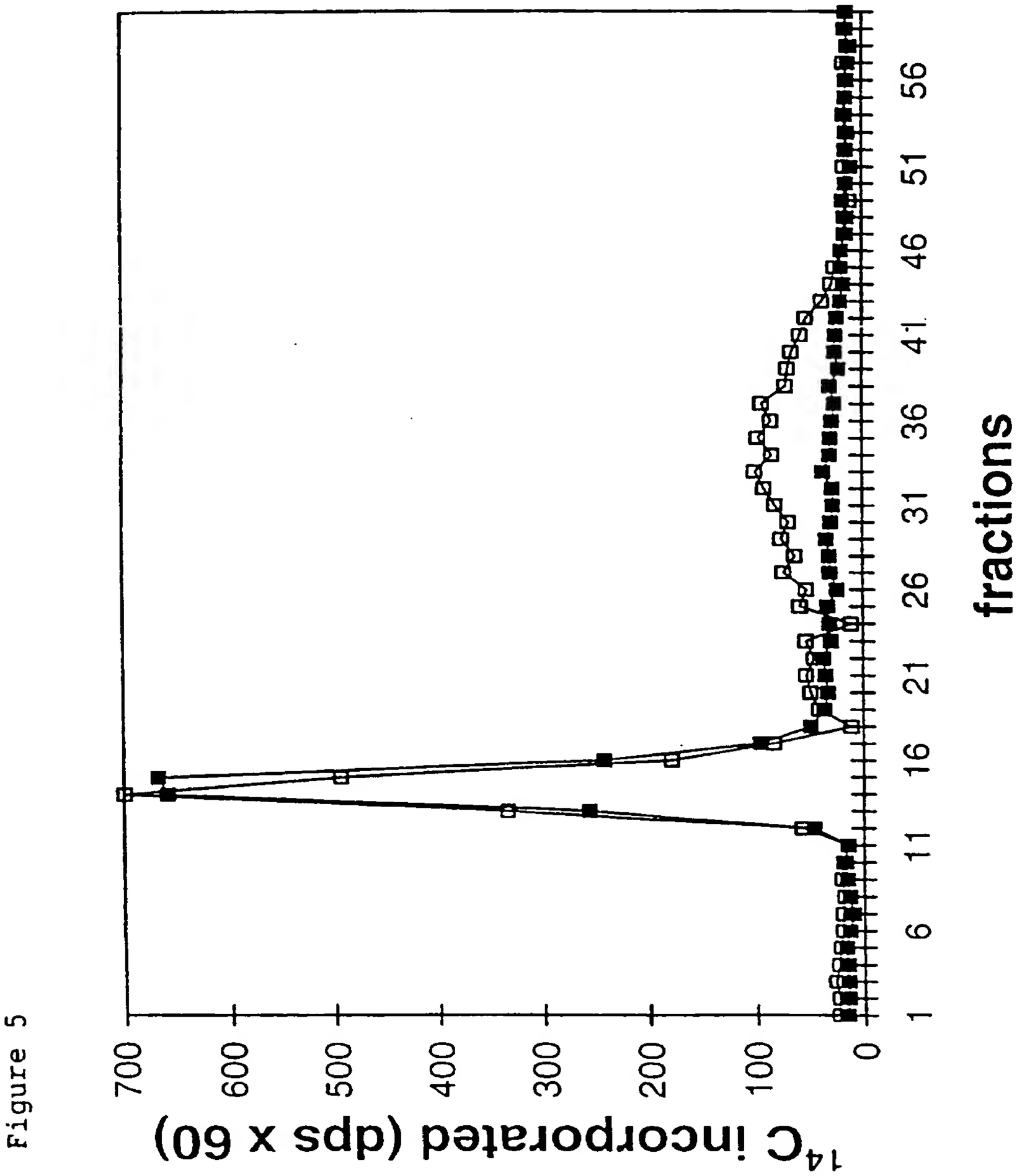
Figure 3

Figure 4

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^{14}C incorporated (dps x 60)
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Figure 5

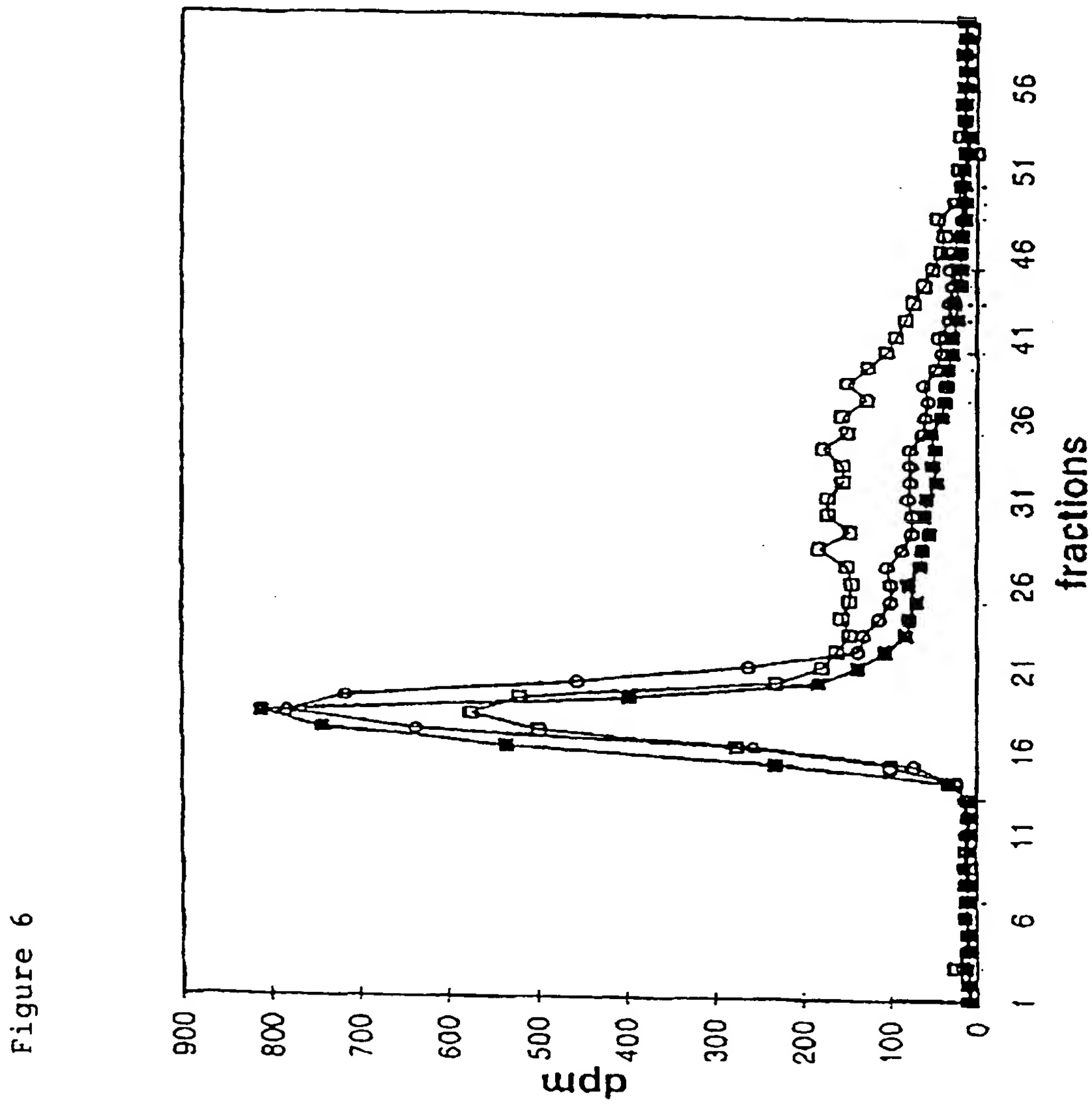
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Figure 6

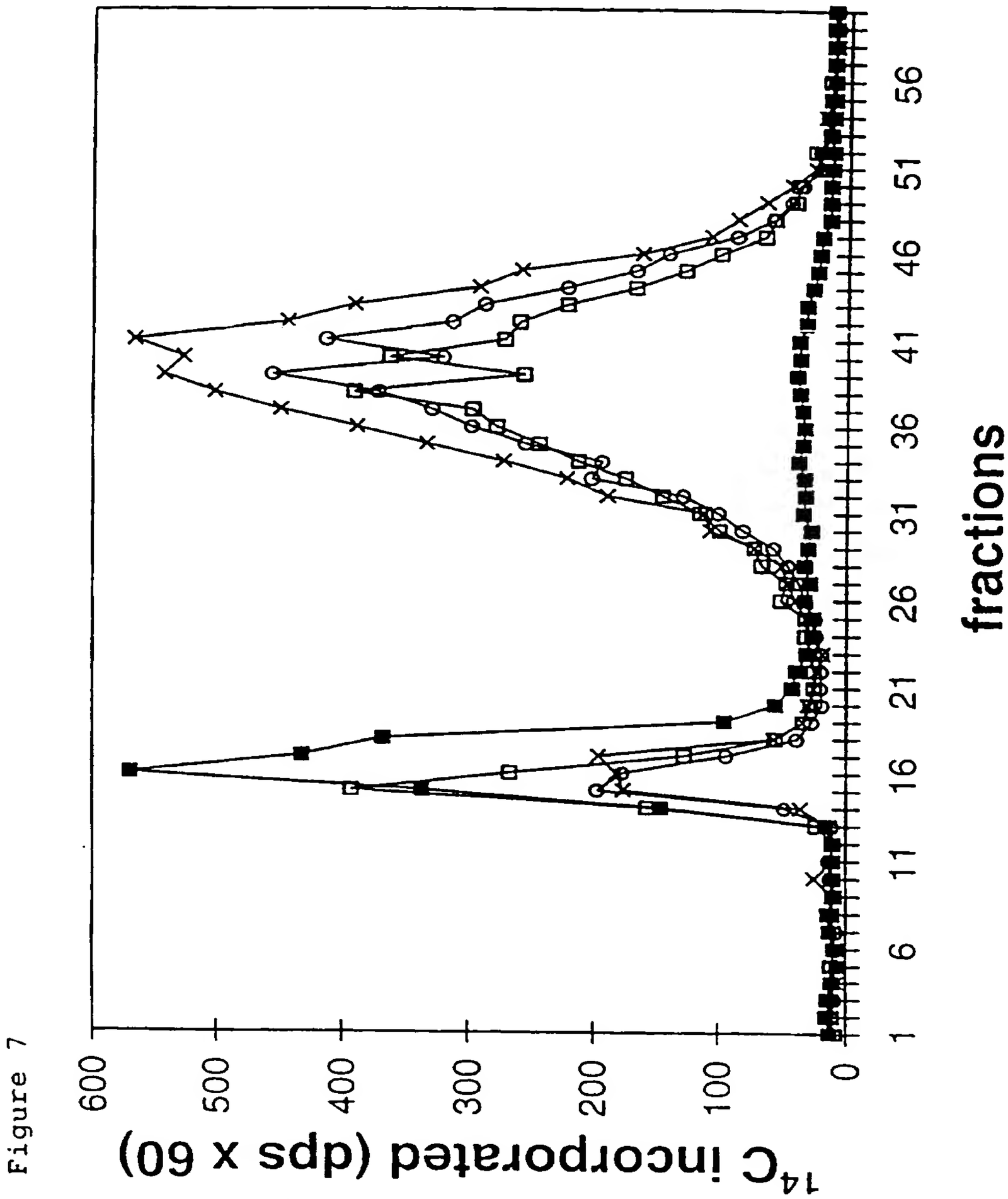
^{14}C incorporated (dps x 60)
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Figure 7

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Figure 8

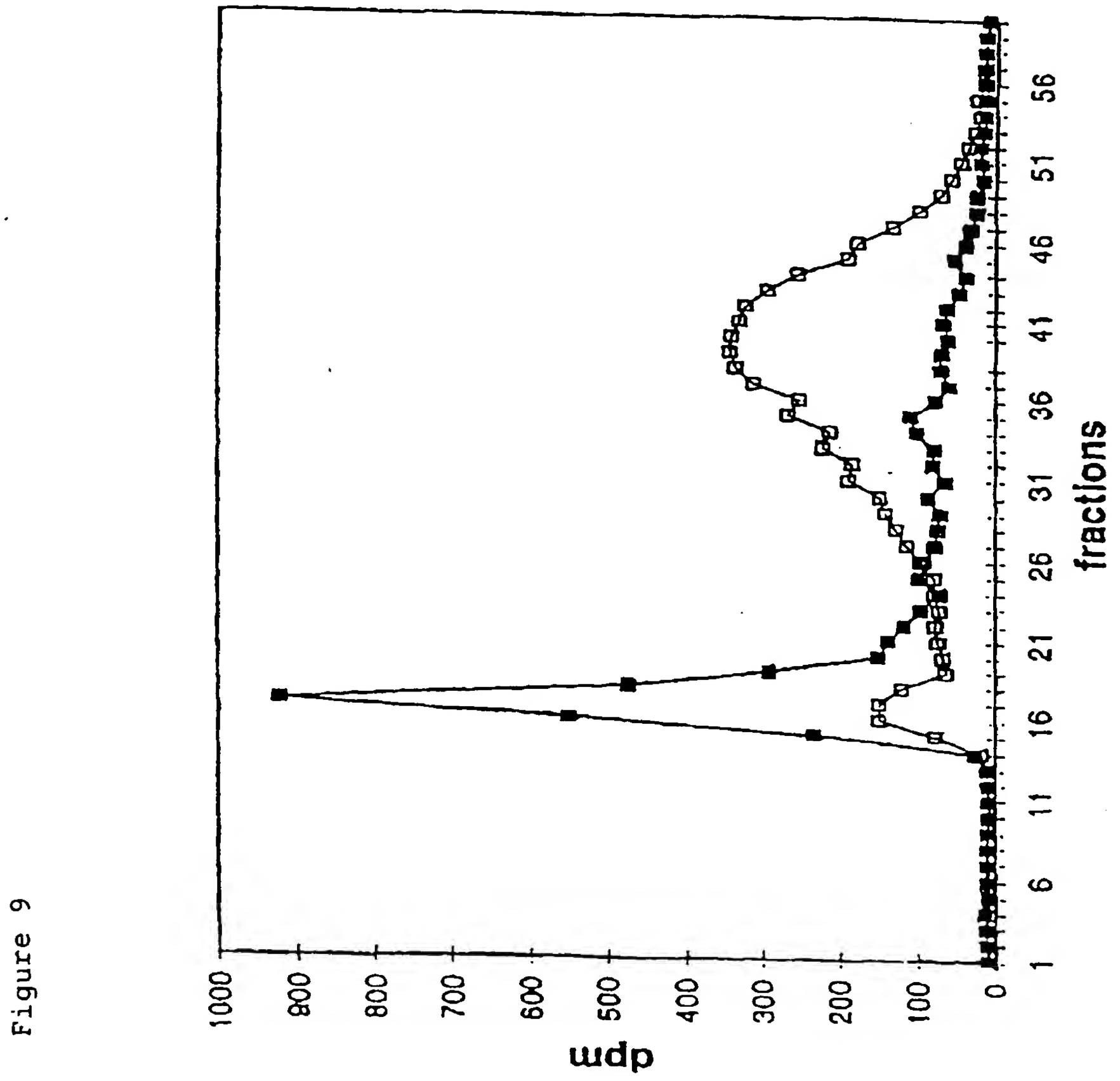
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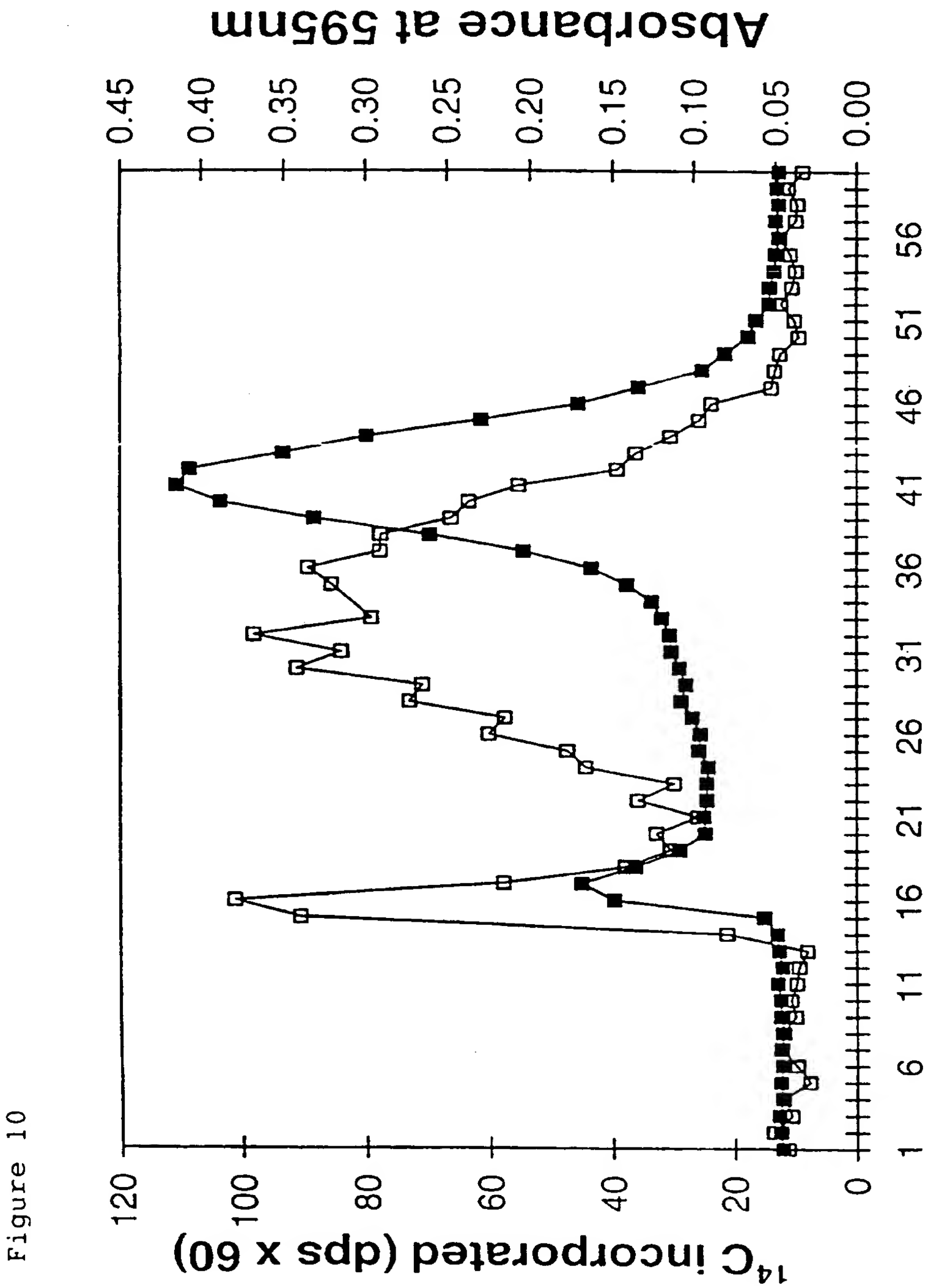
Figure 9


Absorbance at 595nm
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fractions
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40
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^{14}C incorporated (dps x 60)

Figure 10

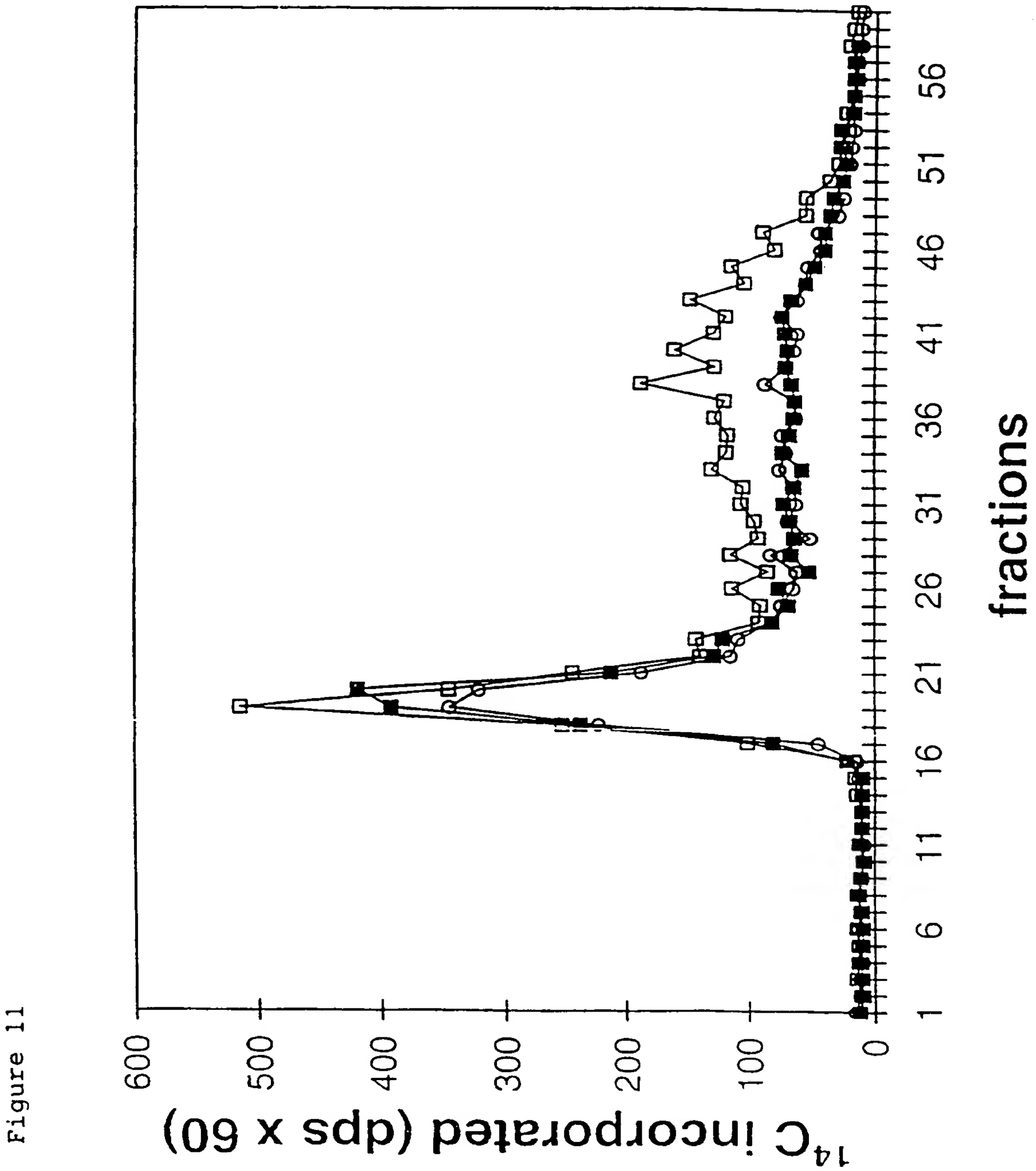
^{14}C incorporated (dps x 60)
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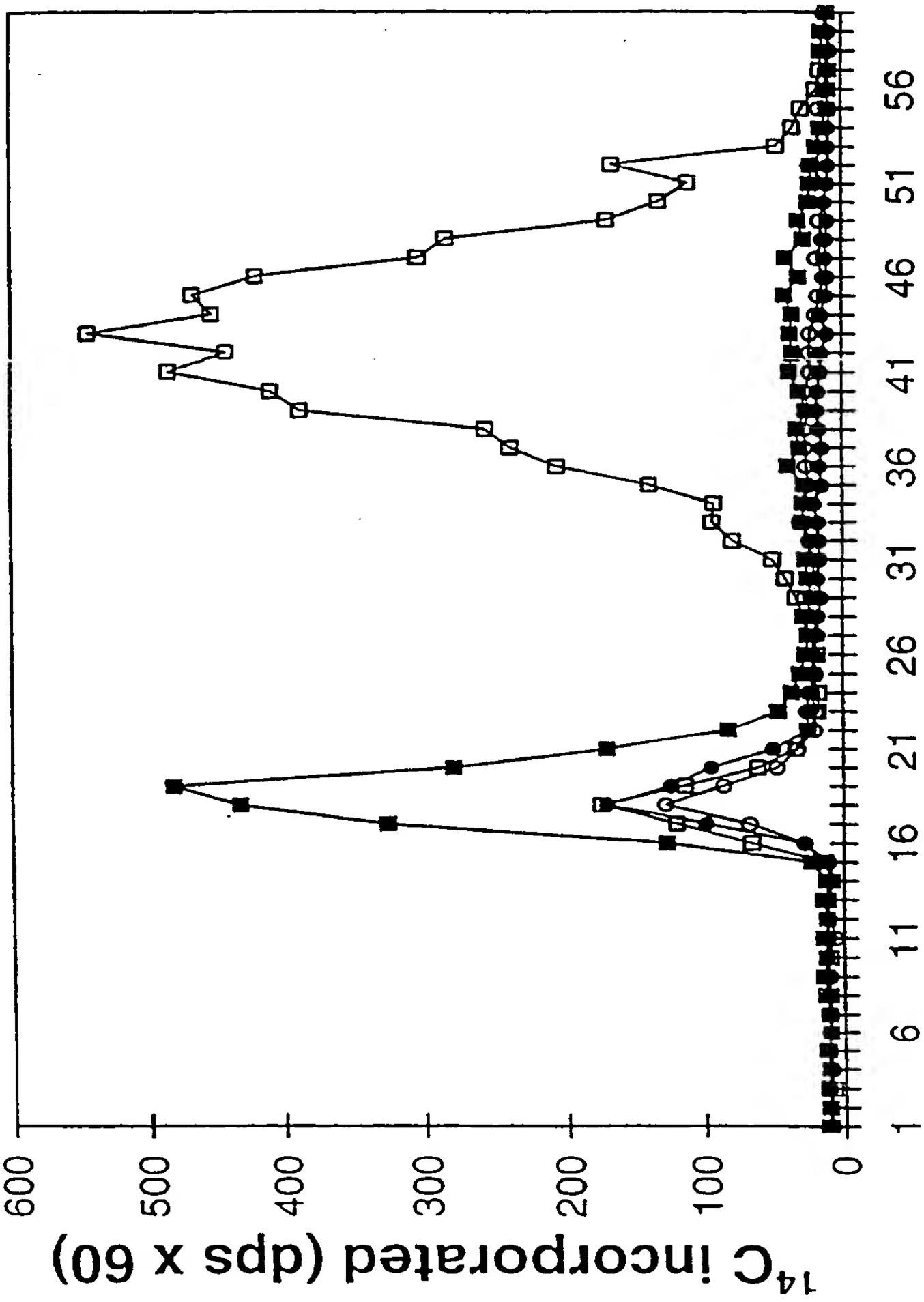
Figure 11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56

Figure 12

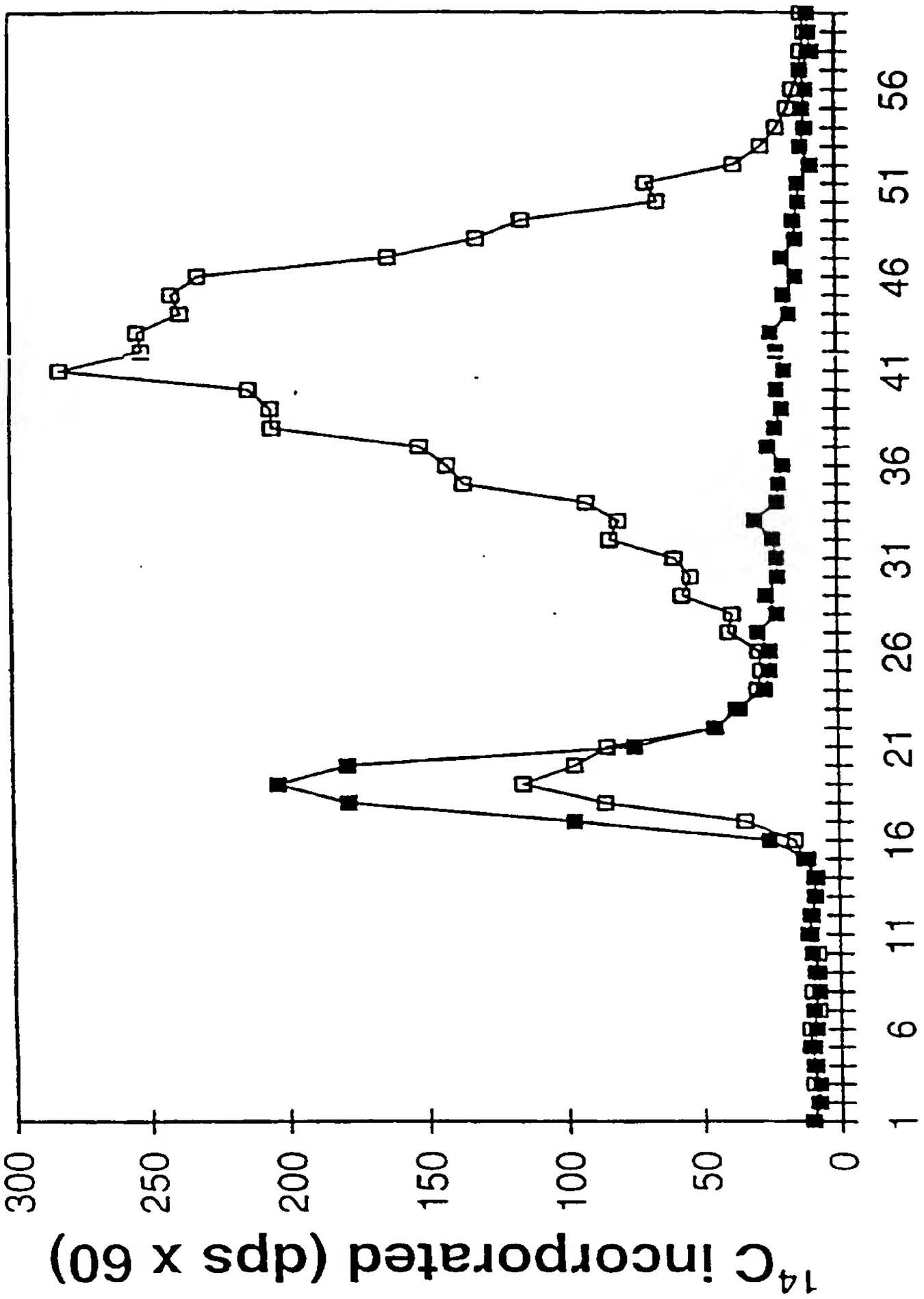
^{14}C incorporated (dps x 60)
300
250
200
150
100
50
0
fractions
1
6
11
16
21
26
31
36
41
46
51
56

Figure 13

INTERNATIONAL SEARCH REPORT

[redacted] onal Application No
[redacted]/GB 96/02696

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/54 C12N15/82 C12P19/04 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12P A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 479 359 A (GIST BROCADES NV ;MOGEN INT (NL)) 8 April 1992 see the whole document ---	1,2,20, 23,26, 28-33, 36-39, 41-44, 48-50, 52,56-68
X	WO 95 04826 A (INST GENBIOLOGISCHE FORSCHUNG ;KOSSMANN JENS (DE); EMMERMANN MICHA) 16 February 1995 see the whole document ---	50
X	WO 91 19808 A (CALGENE INC) 26 December 1991 see the whole document ---	56-62

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 February 1997	07.03.97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	**Authorized officer** Maddox, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 96/02696

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 236, no. 3, 1961, pages 636-64, XP000644719 LELOIR, L.F., ET AL.: "Starch and oligosaccharide synthesis from uridine diphosphate glucose" see the whole document	1-68
A	WO 94 04693 A (ZENECA LTD ;KEELING PETER LEWIS (US); LOMAKO JOSEPH (US); GIEOWAR) 3 March 1994 see the whole document	1-68
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A	THEOR. APPL. GENET., vol. 88, 1994, pages 369-375, XP002021173 FLIPSE, E., ET AL.: "Expression of a wild-type GBSS gene introduced into an amylose-free potato mutant by Agrobacterium tumefaciens and the inheritance of the inserts at the microporic level"	1-68
T	PLANT JOURNAL 10 (6). 1996. 1135-1143. , XP002025748 DENYER K ET AL: "The elongation of amylose and amylopectin chains in isolated starch granules." see the whole document	1-68

1

INTERNATIONAL SEARCH REPORT

[redacted]tion on patent family members

[redacted]onal Application No
[redacted]/GB 96/02696

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